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INACTIVATION OF VIRUSES IN WATER BY BROMINE AND ITS COMPOUNDS:

Influence of Virion Aggregation

FINAL TECHNICAL REPORT

by

D. G. Sharp, Ph.D. and J. Donald Johnson, Ph.D.

February 1977

(For the period 1 August 1973 to 31 July 1976)

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Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Washington, D. C. 20314

Contract Monitor: Mitchell Small, Environmental Protection Division
U. S. Army Medical Bioengineering Research and Development Laboratory

Contract No. DAMD-17-74-C-4013

University of North Carolina
Chapel Hill, North Carolina 27514

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SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE	READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO.
3. RECIPIENT'S CATALOG NUMBER	rept.
4. TITLE (and Subtitle)	(6) INACTIVATION OF VIRUSES IN WATER BY BROMINE AND ITS COMPOUNDS: Influence of Virion Aggregation,
5. ~~TYPE OF REPORT & PERIOD COVERED~~	(9) Final 1 Aug [redacted]73-[redacted]31 Jul 76, [redacted]
6. PERFORMING ORG. REPORT NUMBER	
7. AUTHOR(s) (10)	D. Gordon Sharp, Ph.D. J. Donald Johnson, Ph.D.
8. CONTRACT OR GRANT NUMBER(s)	(15) DAMD-17-74-C-4013, √EPA-R803771
9. PERFORMING ORGANIZATION NAME AND ADDRESS	University of North Carolina, School of Medicine Chapel Hill, N.C. 27514
10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS	
11. CONTROLLING OFFICE NAME AND ADDRESS	
12. REPORT DATE	(11) 15 Feb 1977
13. NUMBER OF PAGES	(12) 120p.
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)	
15. SECURITY CLASS. (of this report)	
15a. DECLASSIFICATION/DOWNGRADING SCHEDULE	
16. DISTRIBUTION STATEMENT (of this Report)	DISTRIBUTION STATEMENT A Approved for public release; Distribution Unlimited
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)	
18. SUPPLEMENTARY NOTES	

ix

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

VIRUSES, treatment with bromine in water.
Bromine for virus inactivation.
Virion aggregation and disinfection
Physical state of viruses in water.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

In this ~~This is an~~ account of the inactivation of poliovirus and reovirus by bromine in water, Emphasis ~~has been~~ is placed on ~~two~~ 2 major points which have received insufficient attention in previous investigations. ~~of this kind.~~ In all experiments the species of bromine, compound or ion acting on the virus has been established and its concentration given and in all experiments the nature and degree of virion clumping or aggregation has been determined and, in most cases, quantitatively. (cont on p ii)

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described. A few experiments on ECHO virus are included.

Methods for direct observation and measurement of the size and frequency of aggregates by electron microscopy have been applied first with reovirus then new methods were devised for obtaining the same data for the smaller polio and ECHO viruses. Methods and apparatus were devised for following disinfection rates at 1/2 second intervals during the fast reactions of some of the bromine species with water suspensions of single virus particles.

Methods have been devised for prepar~~ation of~~ing and storing the viruses with little or no particle aggregation, for storage of such stock preparations with minimal aggregation and for dilution of these stock viruses in water without producing aggregation in the process. These methods have made possible the comparison of the reaction rates of OBr^-, Br_2, HOBr, and the bromamines NBr_3 and $NHBr_2$ on water suspensions of single poliovirus particles. Data have been gathered over a range of concentrations of the active agents, temperatures and pH values. They cannot be abstracted in a sentence but, in general, Br_2 and OBr^- inactivate poliovirus 3 to 10 times faster than HOBr in equimolar concentrations. Tribromamine reacts at about the same rate as the HOBr and dibromamine much slower.

With minor exceptions, the semi-log plot of surviving infectivity of poliovirus in the presence of all of these forms of bromine is a linear function of time but the reaction rate generally does not increase linearly with increasing concentration of the disinfecting agent. Careful investigation with HOBr has shown that its rate of inactivation of poliovirus increases but very slowly when its concentration is increased above 10 μM (1.6 mg/l) at a temperature of 2C. It levels off much like an enzyme reaction as described by the Michaelis-Menton hypothesis, but there are no enzymes involved here.

Aggregation among the virions of both polio and reoviruses increases their resistance to inactivation by bromine. Survival may be increased over 100 fold by this means. Stock virus preparations tend to aggregate when diluted in water of low salt content (natural water) or at pH below 7. Electron microscope data are presented to show this and 2 methods are described for detecting and measuring the extent and progress of this aggregation at virus concentrations too low for electron microscopy. Data obtained by these means show that poliovirus, once aggregated, does not disperse when carried to high dilution in water but addition of salts tends to break up the aggregates. Aggregates of reovirus are more difficult to disperse. Salts are effective in preventing the aggregation of dispersed virus below pH 7 particularly the divalent Ca^{++} and Mg^{++} ions.

Reovirus infectivity is destroyed by HOBr about 15 times faster than poliovirus but if the reovirus is aggregated it can survive HOBr treatment that destroys dispersed poliovirus.

Reovirus, poliovirus and ECHO virus, when released by alternate freezing and thawing of a few infected cells in distilled or natural water, are not well dispersed in spite of great dilution. Ten to 50% of the infectivity is in the form of aggregates. This is in water without particulate impurities. Clay particles (bentonite) attract reovirus in weak phosphate buffer at pH 7 as shown by electron microscopy.

Aggregated appears to be the common condition of these viruses when present in water of zero or low salt content or at low pH. And the resistance to disinfection by bromine is greatly increased by aggregation.

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ACKNOWLEDGEMENTS

While the major part of the funds for support of this research came, as indicated on the cover page of this report, there was other support from EPA research grant R803771 which began during the second year of the work and is continuing now. It is this increased and continuing EPA support that should bring to fruition some of the projects that have been started here.

Two people have performed most of the experiments, Roger Floyd Ph.D. and Dorothy C. Young MS. They have produced the data and to them should go the credit for good work done. We are also much appreciative of the excellent technical work of Florence Stubbs, electron microscopist and David Hayes for preparation and titration of the bromine solutions.

Dr. Richard Sceares of the NASA-sponsored Research Triangle Institute Biomedical Applications Team located at Research Triangle Park, N.C. and Mr. Richard Boynton of the Sperry Rand Co. joined efforts that provided the special mixing motors for the Flash Flood experiment. All other items of equipment were designed and built by the author in his home machine shop.

INTRODUCTION

The most practical method in use today for inactivation of both bacteria and viruses in water is treatment with halogens. No publication of any consequence has denied their effectiveness on viruses but few have been in good agreement regarding the rate of inactivation and most experiments have encountered a departure from simple first-order kinetic behavior. Our work has not been directed to discovery of the nature of the chemical reaction of halogen compounds with viruses but rather to make a careful study of the kinetics of their inactivation by the important individual chemical forms of bromine with simultaneous observation of the aggregation of the virus particles. Many authors in the past have cited aggregation as a probable cause of departure from classical kinetic concepts (1) so we have made direct measurements of both the number of virions (physical particles) and their state of aggregation by electron microscopy. Special quantitative techniques have been devised and developed for this purpose. And these have been supplemented by analytical procedures involving rate zonal centrifugation which are effective at very low virion concentrations where electron microscopy (EM) does not apply.

Inasmuch as bromine has been the particular agent under study it has been necessary to depart from the conventional procedure of mixing virus and halogen in a beaker and taking samples for titration at suitable times. The reaction with single virions is so rapid, even at 1 μM (0.16 mg Br_2/l) concentration of HOBr that it could not be adequately followed in such a static experimental arrangement. A dynamic experiment has been designed for injecting stock virus into a turbulent flowing stream of bromine water and removing samples for test at appropriate intervals down-stream that can be made to correspond to treatment times as short as one half second. A description of apparatus for this as well as for the preparation of virus for the EM analyses is a significant part of this report.

While data on the survival of aggregated virus has been sought and obtained here, it has been necessary to learn first how to prepare and store purified virus stock suspensions that have essentially all single particles. These must have high enough concentration for quantitative electron microscopy and they must not aggregate on dilution during the experimental procedures. Without such dispersed starting stocks of virus, no meaningful comparisons can be made of reaction rates for different viruses or for different bromine species on a given virus which sometimes require different pH values. We believe we have satisfied these conditions and that the reaction rates given here are indeed those for single virions.

Halogen disinfecting agents have sometimes been used in biological experiments in such a way that one cannot be sure of the concentration or chemical form of the active component. We have been careful to not only control and quantitate the state of viral aggregation but also have carefully controlled the concentration and chemical form of the halogen used. In this way we have developed our kinetics of disinfection with both known and reproducible chemical and viral conditions.

MATERIALS AND METHODS

THE VIRUSES, (THEIR PREPARATION AND ASSAY)

Most of the experiments reported here were done with two viruses. Reovirus, although often considered of little significance as a disease producer in water, was important to us because of its size. It is 2.5 times larger in diameter and so about 16 times as massive as polio or any of the small enteric viruses. This virus could be counted and its aggregation measured by existing quantitative methods of electron microscopy (2). Methods for doing this with polio and other such small viruses had not yet been developed to the point of general usefulness at the time we began this work. So reovirus has been a valuable intermediate in the transition to the extended work with polio and the final brief series of experiments with ECHO.

Reovirus type 3 (Dearing strain) was grown in and all plaque titrations were made on monolayer cultures of L cells as previously described (3), with the following exceptions: Cells were maintained in 200 ml milk dilution bottles and passaged into 32 oz prescription bottles for growth of virus. Virus was harvested at 16 to 18 h after infection at 10-20 pfu/cell rather than 20 to 24 h as previously noted (3). In addition, the virus was extracted from the cells in 6 ml of PBS without calcium and magnesium and 4 ml of Freon 113 for 2 min at one half speed in a Sorvall Omni-Mixer. The virus was placed on 20 → 40% w/w sucrose gradients in 0.05 M phosphate buffer at pH 7.2, and centrifuged at 25,000 RPM in the Beckman SW 27 rotor at 4C for 1 hour. The virus, collected from the lower of two bands seen with a collimated beam of light, was allowed to remain in the sucrose at 4-6C, and was not pelleted. The virus stored in this manner retained its infectivity for several weeks and the state of physical aggregation is quite stable. There is no bacterial or fungal growth and the sucrose does not produce any detectable bromine demand under the conditions of our experiments.

Poliovirus, type I, Mahoney strain, was obtained from Dr. Gerald Berg, Environmental Protection Agency, Cincinnati, Ohio, and was serially passaged in Human Epidermoid Carcinoma cells, HEp-2. The cells were grown in medium 199 containing 0.105% $NaHCO_3$ and 5% fetal calf serum (FCS). After the cells reached confluency they were maintained on the same medium with 2% FCS.

Stocks of virus were produced in HEp-2 cells at 37C by infection at a multiplicity of 10-20 pfu/cell under a maintainence medium of 199 + 0.105% $NaHCO_3$ and 2% FCS. After 18-24 h when the CPE was 100%, the cells were frozen and thawed 3 times and the cell debris removed by low-speed centrifugation (~800G) for 10 min. The supernatant fluids had titers of 4×10^{10} PFU/ml and were kept frozen at -70C.

Purified virus was produced in the same cells grown for 3 days in 32-ounce prescription bottles inoculated with the above stock virus in Dulbecco's PBS containing 12.5 mM $MgCl_2$, at a multiplicity of ~10 PFU/cell. The virus was allowed to absorb for 1 h at 37C, then 40 ml of maintainence medium containing 12.5 mM $MgCl_2$ was added to each bottle and the cells were further incubated at 37C for 11 h. The cells were chilled to 4C and the supernatant fluid was decanted. The monolayers were washed 2 times with PBS and the cells of each bottle were scraped into 10 ml of PBS and pelleted at 250 G for 10 min. Cells remaining in the supernatant fluid were pelleted similarly and pooled with the scraped cells and pelleted again. The pellet of cells was resuspended in 6 ml of PBS not containing Mg^{++} or Ca^{++}. Four ml of Freon 113 were added and the cells were homogenized at one-half speed in a Sorvall Omni-Mixer for 2 minutes. The phases were separated at 800G for 10 minutes and the upper aqueous phase was removed and held in an ice bath.

The Freon phase was re-extracted with a further 6 ml of PBS and the phases again separated. The aqueous phase was pooled with the previous one, and the Freon phase again re-extracted with 6 ml of PBS. All aqueous phases were combined and made to a volume of 20 ml. Ten ml of this extract was placed on each of two 10 → 30% w/w sucrose gradients made in 0.05 M phosphate buffer, pH 7.2. The gradients were centrifuged at 25,000 RPM in the Beckman SW 27 rotor at 4C for 2 hours and 15 minutes. Fractions of 2 ml each were collected from the tubes and examined by the kinetic attachment (4) for the presence of the virus. Twenty fractions were obtained from each tube and the highest count of virus was most frequently found in fraction 14. All relevant fractions were pooled and the virus was stored at refrigerator temperature without any attempt to remove the sucrose.

ECHO was grown in HEp-2 cells, purified and stored exactly the same as poliovirus.

PLAQUE ASSAY

Plaque titrations of reovirus were performed on 3 day old monolayer cultures of L cells (5) in tightly stoppered 1 oz prescription bottles under an overlay of 1% Difco agar containing Medium 199, 5% Fetal Calf Serum, 0.245% $NaHCO_3$, and 0.003% Neutral red. Plaques were counted after 6 days at 37C.

Plaque assays of poliovirus were performed on 4-day-old monolayers of HEP-2 cells in 1 ounce prescription bottles under an overlay of 1% Difco agar containing medium 199 plus 5% FCS, 0.210% $NaHCO_3$, 0.003% neutral red, and 5 mM $MgCl_2$. The plaques were read after 3 days incubation at 37C.

Plaquing of ECHO-1 virus was done the same as that of poliovirus.

PHYSICAL ASSAY (Particle counting and Aggregation analysis)

Physical assay of virus particles in suspension by electron microscopy can be done by the method of Sharp (2) if the virions are large enough and reovirus is large enough (3). Briefly the method consists of sedimenting the virions from 1ml of water-suspension upon one cm square piece of 2% Difco nutrient agar 2 mm thick in a Sorvall Type SU centrifuge rotor. When all the virions have made contact with the agar the machine is stopped, the supernatant fluid poured off and the agar block permitted to dry (15 minutes). After drying, a 0.75% solution of collodion in amyl acetate is flooded over the surface of the agar block which is immediately drained edgewise (with one edge resting on an absorbant paper). This removes most of the solution leaving a very thin film which dries quickly. This collodion film is then removed by floation on a clean water surface and mounted, virus side up, on standard EM grids which may be shadowcast with metal to enhance contrast and to reduce the eyestrain of particle counting. Collodion films so prepared are not true replicas but pseudoreplicas. The reovirus particles that were on the agar surface adhere to the collodion and are removed with it. The number of virions per unit area of the picture N_c is related to the number per unit volume N_o as follows:

$$N_o = \frac{N_c M^2}{k}$$

in which k is a constant determined by the geometry of the centrifuge rotor cell and the thickness of the agar and M is the magnification in the EM. In our apparatus k = 21 so when M = 2500 there are 3.36 virus particles on each 2" square picture per million in the ml of fluid examined. Inasmuch as about 100 per field are desirable for statistically significant results our practical lower limit of sensitivity is about 3×10^7 virions per ml which corresponds to about 7×10^5 plaque forming units (PFU) of fully active reovirus. Naturally one makes these count pictures at as low a magnification as possible, consistent with ability to see and focus the instrument, in order that the maximum number of particles be seen.

Visual counting and aggregation analysis of poliovirus was essential to this work so our first efforts were directed to application of the agar pseudoreplica sedimentation technique to these 28 nm particles. It failed. But another method was devised, based on work done by Valentine and Allison in 1959 (6). Brownian motion of virions in a liquid in contact with a suitable surface will deposit on that surface a predictable number of particles per unit time. Enteric virus particles like polio, coxsackie, ECHO, etc., are small and so they diffuse rapidly. Apparatus was constructed for holding a drop of virus suspension, without evaporation, on an aluminum-coated collodion film supported by a standard EM grid (Fig 1). After a suitable contact time, usually less than one hour, all the liquid is washed away. This washing is very critical. Absolutely no unattached virus must remain to dry down on the surface because the deposit by drying will not be random. After drying, the preparation may be metal shadowcast. This is not essential but it helps greatly in seeing and focusing at very low magnifications and in the tedious business of counting thousands of particles on the negatives. At M = 5000 suitable count pictures of poliovirus can be made and the number of particles Nc that can be seen per unit area from a suspension of No per ml. will be:

$$N_c = 1.13\, N_o \sqrt{Dt} / M^2$$

in which D is the diffusion constant which is given by:

$$D = kT/6\,\pi\eta r$$

$k = 1.38 \times 10^{-16}$ (Boltzman's constant)
T = Absolute temperature
η = Absolute viscosity of the fluid at temperature T.
r = Particle radius (14×10^{-7} cm for poliovirus)
For poliovirus $D = 1.53 \times 10^{-7}$ at 20C.

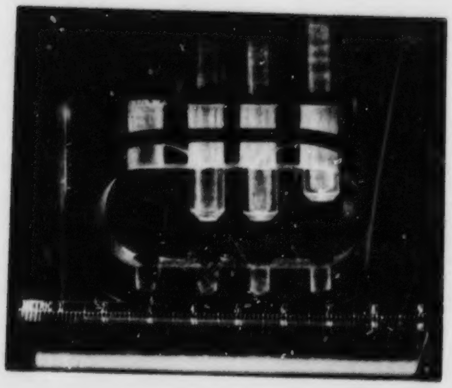

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Fig. 1 KA apparatus for producing random kinetic attachment of single virions and clumps to an aluminum-coated colection film for assay by electron microscopy.

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When deposit time has been 30 minutes at 20C and EM magnification 5000 there will be about 2 particles per 2" square picture for each 10^8 per ml in the virus suspension so 5×10^9 per ml are needed to get 100 per frame compared with 3×10^7 for reo by spin-down methods. We have called this the kinetic attachment or KA method and the sedimentation one, the spin-down or SD method in the rest of this report. Both are satisfactory for reo but only the KA succeeds with polio and ECHO viruses. Three more details are important to those who would use the KA method. Salt concentration in the virus suspension had best be above 0.1 M; sucrose up to 3% is tolerable but soluble protein must be rigidly excluded. The method will not work except with purified virus from which proteins from host cells and culture media have been removed.

HALOGEN SOLUTION, PREPARATION AND ANALYSIS OF INDIVIDUAL CHEMICAL COMPOUNDS (The separate chemical species used in dynamic inactivation experiments)

Chlorine demand free water was prepared from deionized, glass-distilled water chlorinated to about 5 mg/l for 4 [illegible] dechlorinated the day before the experiments with an ultraviolet [illegible] This water referred to as demand free water was used in the preparation of all solutions and reagents coming in contact with halogen solutions. It is stored protected from the atmosphere by 50% sulfuric acid scrubbers.

Hypobromite stock solutions, of approximately 8 mM concentration, were prepared by the reaction between hypochlorite and bromide (7), at pH 11.0, using equimolar amounts of NaOCl and KBr. Because of the slow decomposition (8,9,10,11), yielding bromate and bromide, stock solutions were used for a maximum of one month. To monitor decomposition, a differential titration procedure for hypobromite and bromate was devised. Excess sodium arsenite was added to two stock samples; one in acidic and the other in neutral media followed by an amperometric back titration of each, with bromate and iodine, respectively. Since bromate is determined only in the acid titration, while hypobromite is determined in both, bromate concentration is calculated by difference, indicating the magnitude of stock decomposition (12,13,14,15).

Hypobromous acid, HOBr, was produced by adding the stock solution to pH 7 buffer (KH_2PO_4-K_2HPO_4, 0.01 M) just prior to the experiment. As in all experiments, including those involving acetate and borate buffers for other bromine species, the buffer was chlorinated to 3-5 mg/l for 4 days and dechlorinated with an ultraviolet lamp the day before the experiments.

Hypobromite, OBr^- was also produced by adding stock solution to the carboys immediately before the experiments. One set of experiments was done at pH 10 (0.01 M $Na_2B_4O_7$, NaOH), the other at pH 11 (0.001 M NaOH). Equillibrium calculations show that at pH 11, the solution at 4°C is 99% OBr^- and 1% HOBr, while at pH 10, it contains 91% OBr^- and 9% HOBr (16).

Molecular bromine, Br_2, was produced by adding an OBr^-/Br^- mixture (pH 11) to a buffered pH 5 solution (0.05 M CH_3COONa - CH_3COOH). In each experiment, the final bromide concentration was 5.4 mM, such that the final solution at 4°C was composed of 89% Br_2, 10% Br_3^-, and 1% HOBr (17). In addition, NaCl was added to the buffer to a final concentration of 0.3 M in order to prevent viral aggregation.

Tribromamine, NBr_3, was produced by simultaneously adding equal volumes of OBr^- and NH_4Cl solutions to pH 7 buffer, (KH_2PO_4-K_2HPO_4, 0.01 M). The resulting solution had a $[NH_3]: [Br_2]$ of 1:3, such that the bromine existed entirely as tribromamine (18).

Dibromamine, $NHBr_2$, was also generated by simultaneous addition of equal volumes of OBr^- and NH_4Cl solutions to pH 7 phosphate buffer, 0.01 M. However, the final ratio $[NH_3]:[Br_2]$ was 3:1 (18). Both the di- and tribromamine experiments were conducted within several minutes after preparing the solutions. All experiments were done at 4°C.

BROMINE ANALYSIS

Bromine concentrations were determined by the iodometric method (19). Immediately before and after each dynamic inactivation experiment, flow output samples were collected into 250 ml optical red flasks, which already contained 5 ml. of 5% potassium iodide. Then 2 ml of glacial acetic acid was added, and the solution was titrated with sodium thiosulfate to an amperometric endpoint, using an applied potential of + 200 mV vs SCE.

In addition to iodometry, ultraviolet absorption spectroscopy was used in the analysis of di and tribromamine. Ultraviolet absorption spectra of samples from each experiment were taken on a Cary model 14 spectrophotometer in 10 cm cells. Simultaneous Beer's law equations involving the molar absorptivities of di- and tribromamine at their absorbance maxima of 232 and 258 nm (20) were then solved, showing that each sample was essentially either all dibromamine or all tribromamine, with only minute traces of the other.

Dynamic Inactivation Experiment

(For exposing virus to bromine in turbulent flowing water)

Suspensions of single virions react rapidly with bromine even at very low concentrations so apparatus was constructed in which a virus stock suspension could be injected into a turbulent flowing stream of bromine water at measured rate for a short time then sampled at appropriate distances down-stream. This minimized the quantity of virus needed (virus production is time-consuming and expensive) while providing samples of very short but precisely known exposure times. The apparatus must provide a large enough volume of water to insure stability of temperature, bromine concentration and flow rate through the reaction siphon tube (Fig. 2) for a period of several minutes. The reaction tube is 3/8 inch inside diameter and the bottle (20 liter) height is adjustable so that turbulent flow at the desired rate can be achieved. Turbulence is essential, so the Reynolds Number has been kept equal to or greater than 3000 in all experiments. The reaction tube has 5 access ports covered by disposable serum bottle stoppers. Five 5 ml syringes with needles are inserted. The first of these (at the left) carries the virus inoculum (5 ml) which, when triggered, starts the weight-driven plunger, which delivers the inoculum steadily for 5 seconds, into the turbulent stream of buffered bromine water. There is a 6 mm diameter disk located on the axis of the tube just downstream of the inoculum needle tip, to aid in mixing.

The time of transit from injection point to sampling point will be determined only by the flow rate and the distances D_1, D_2, etc. It is not in any way dependent on when the sample is taken as long as the sample is taken within the 5 second interval during which the "polluted" water passes the sampling port in question.

Each of the 4 sampling syringes contains 1 ml of 2 mM autoclaved, membrane filtered sodium thiosulphate solution, 1 ml of air and a small Teflon-coated magnetic stirring bar. The syringe plungers are held in metal blocks attached to helical springs, each under tension and restrained by a release pin that can be pushed at the desired time. When the spring is released the syringe draws exactly 1 ml from the flowing stream. This is mixed with the thiosulphate solution to stop the reaction of the bromine on the virus. For virus-bromine exposure times of 1 second or less, additional precautions were taken to insure instant mixing. This is done by means of the magnetic stirring bar which is made to rotate at 1800 RPM by 2-pole AC motor windings surrounding each sampling syringe. These are stator windings used in small electric servo motors kindly provided for us by the Sperry Rand Company of Durham, N. C.

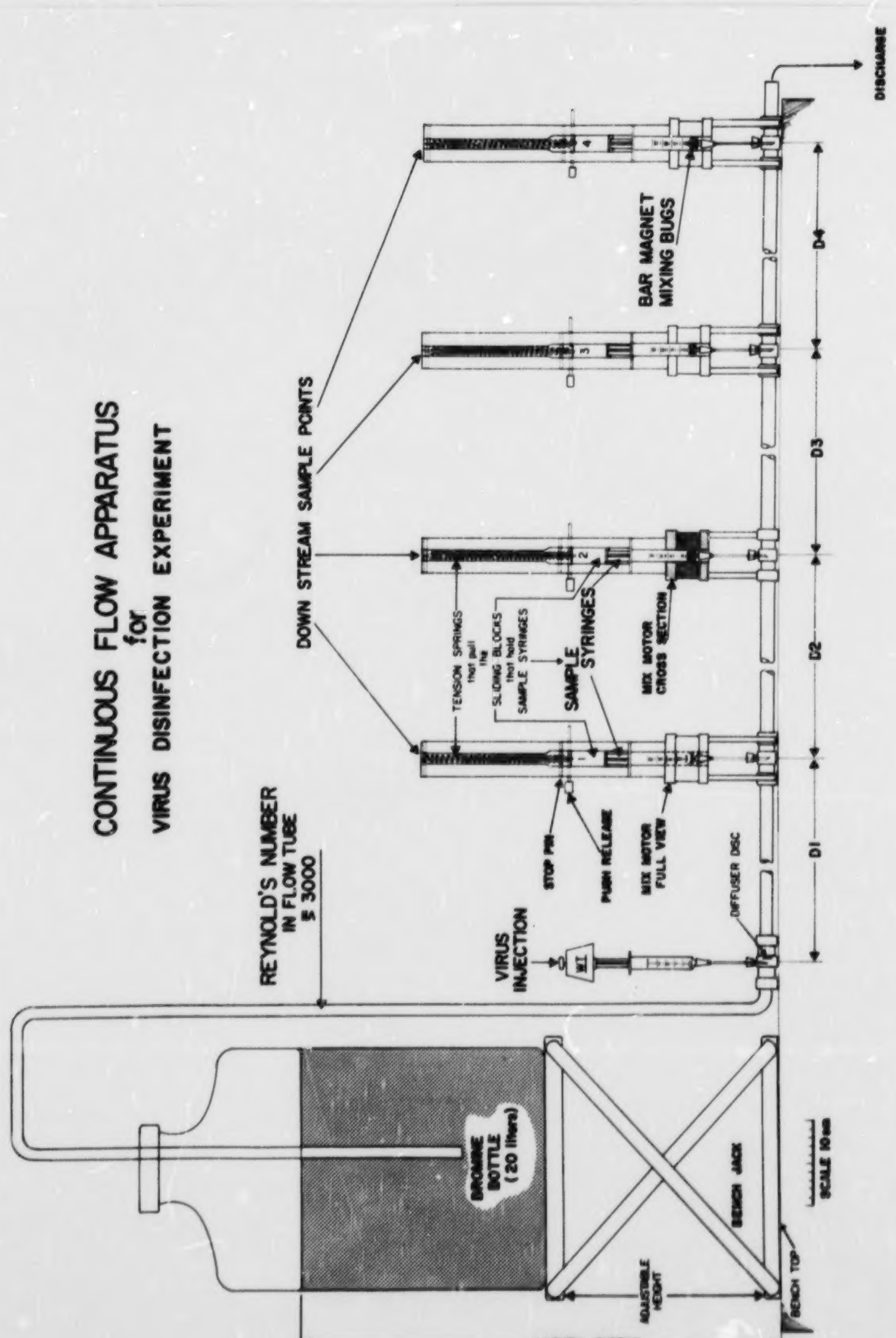

CONTINUOUS FLOW APPARATUS
for
VIRUS DISINFECTION EXPERIMENT
REYNOLD'S NUMBER IN FLOW TUBE ≤ 3000
VIRUS INJECTION
WT
DIFFUSER DISC
STOP PIN
PUSH RELEASE
MIX MOTOR FULL VIEW
TENSION SPRINGS that pull the
SLIDING BLOCKS that hold SAMPLE SYRINGES
SAMPLE SYRINGES
MIX MOTOR CROSS SECTION
DOWN STREAM SAMPLE POINTS
BAR MAGNET MIXING BUGS
1
2
3
4
D1
D2
D3
D4
DISCHARGE
BROMINE BOTTLE (20 liters)
BENCH JACK
ADJUSTABLE HEIGHT
LIQUID HEIGHT
BENCH TOP
SCALE 10cm

Fig. 2
Apparatus for
short-time
treatment
of virus in
water with
bromine.

Twenty liters of buffered demand-free water is prepared and adjusted to the temperature required for the experiment. Enough bromine of the proper chemical form is added 17 hours before the experiment to bring the concentration in the bottle to approximately the right value. Just before the experiment, more bromine is added to adjust the concentration to exactly the required level. Previous experiment has established the exact level of liquid in the bottle to produce the desired flow rate (range 20 to 40 ml/sec in different experiments). This level is marked for easy reference and all experiments start at this level. Beginning with the liquid at a higher level, the siphon is started and the flow continued for a few minutes to allow the tube to adjust to the temperature and concentration in the bottle and when the liquid level reaches the marked starting level the virus injection is started. The stirring bars in all four sampling syringes are already running in their 1 ml volumes of thiosulphate solution and it only remains to trigger each of the syringes as the virus passes and also catch a sample of the virus-bromine mixture at the discharge end to determine its bromine concentration for comparison with that in the bottle. Neutralized bromine-virus mixtures from the sampling syringes and the virus from the inoculation syringe are then assayed for virus by the plaque method.

The dilution of virus, as it enters the flowing stream is just the ratio of the rates of injection and stream flow. The injection syringe is timed by direct observation and stopwatch. The stream flow rate is easily obtained by timing the outflow of some conveniently measured volume such as 500 ml. Variation in flow rate with liquid level in the bottle is negligible during the experiment time, which has not been over 20 seconds, because of the large (approx. 700 cm^2) surface area.

Tygon tubing is convenient for the flowing stream but we have found that it absorbs substantial quantities of bromine. Glass is difficult to manage without catastrophic accidents. However, "high density" polyethylene tubing has performed well. It is readily cut to desired lengths and it exerts negligible bromine demand.

MEASUREMENT OF FREQUENCY DISTRIBUTION OF AGGREGATES

Rate Zonal Centrifugation

Quantitative data on virus particle aggregation from electron microscopy has a unique directness that is appealing but it can be obtained only from suspensions of high concentration. The centrifuge will reveal the differences in sedimentation rate characteristic of single particles and aggregates of single particles no matter how low the concentration might be. It is particularly powerful when employed to sediment a band of virus particles into a shallow preformed gradient of density made of an inert non-ionic solute such as sucrose. This is not an isopycnic equilibrium but a rate-zonal process that can be carried out either in a large-volume "zonal" rotor or a conventional swinging bucket rotor. We have employed them both for different purposes in this work.

ZONAL ROTOR (Analytical technique)

The BXIV zonal centrifuge rotor (23) was brought up to 1,800 rev/min empty and held at this speed while gradient and virus samples were introduced through tygon tubing of one-eighth inch (3.2 mm, internal diameter) via the running seals. Slightly convex gradients were produced with reagent-grade sucrose, in 650 ml quantitites, in 0.005 M phosphate buffer (pH 7.0). These were pumped into the rotor, entering at the periphery, dilute end first, until the entire volume was filled. Virus was introduced (15 ml volume containing about 10^{10} virions) by means of a syringe and needle, placing it in the inner-most position. In the experiments with 10 to 30% sucrose gradients, the virus was suspended in 5% sucrose made up with phosphate-buffered saline. In later experiments with 15% to 30% gradients, the virus was suspended in 15% sucrose made up with 0.005 M phsophate buffer, pH 7.0. A 50 ml volume of buffer was injected afterward, displacing a total 65 ml volume of the denser part of the gradient out through the tubing through which it came in and advancing the virus to occupy a band 1.6 mm thick at the 2.7 cm rotor radius. The running seal with connecting tubing was then removed, and a vacuum-tight cap was put over the opening in the rotor. The centrifuge was evacuated, and the rotor speed was increased smoothly to the desired maximum and held constant until deceleration. Some of the experiments were made with the Spinco model L centrifuge; with it, the rotor was allowed to coast, without brake, down to 1,800 rev/min, when air was admitted to the rotor chamber. In later work with the Sorvall ultracentrifuge, in which there is no gear train to provide frictional drag, a small amount of reverse pressure had to be applied to its fluid drive to decelerate the rotor to unloading speed in about 6 min. Running seal and feed lines were reattached, and the whole contents of the rotor were displaced through the innermost connecting tubing, by pumping in a denser piston fluid through the peripheral connection. Twenty-six bottles (25 ml each) were collected in series in this unloading operation. Densities were determined by 1 ml pycnometer. Virion counts and aggregation analyses were made on suitably diluted samples by the agar pseudoreplica technique with the use of the electron microscope. Most methods of preparing suspended virions for electron microscopy will induce some aggregation, which cannot be tolerated here. Sucrose concentrations of 3% (w/v) or less do not interfere with the virion-counting technique, so a maximum dilution of 10 x was needed only at the 30% end of the gradient. Low-count samples were sometimes dialyzed to avoid dilution before counting. The number of single particles and the number of groups in each size category were counted in each of the samples.

Suitable dilutions of the samples were made directly into growth medium 199 and titrated for plaque formation upon monolayers of L cells.

Swinging Bucket Rotor

A Single Particle Approximation (SPA) test has been used to determine the concentration of single particles in a virus suspension. Figure 3 shows the geometry of the test. A cellulose nitrate tube which fits the Beckman SW 50.1 rotor (1/2 by 2 inches, 1.27 x 5.08 cm) is filled uniformly with the virus sample. Under the centrifugal force developed by the spinning rotor, each aggregate size has a characteristic velocity of sedimentation. Singles sediment slowest, while pairs sediment approximately 40% faster. Groups of three, four, and larger each sediment somewhat faster. If the run is terminated at the proper time, those single particles which were at the meniscus of the fluid will sediment one-third of the distance down the tube. Pairs will sediment about one-half the tube length, while larger aggregates will all enter the bottom half of the tube. Therefore, in that volume of fluid between one-third and one-half the length of the tube will reside single particles only (the shaded area in Fig. 3), and removal and titration of the top half of the contents of the tube (ca. 2.3 ml) will yield a measure of the singles population. The more aggregation present, the fewer singles remaining in this volume of fluid. For poliovirus,

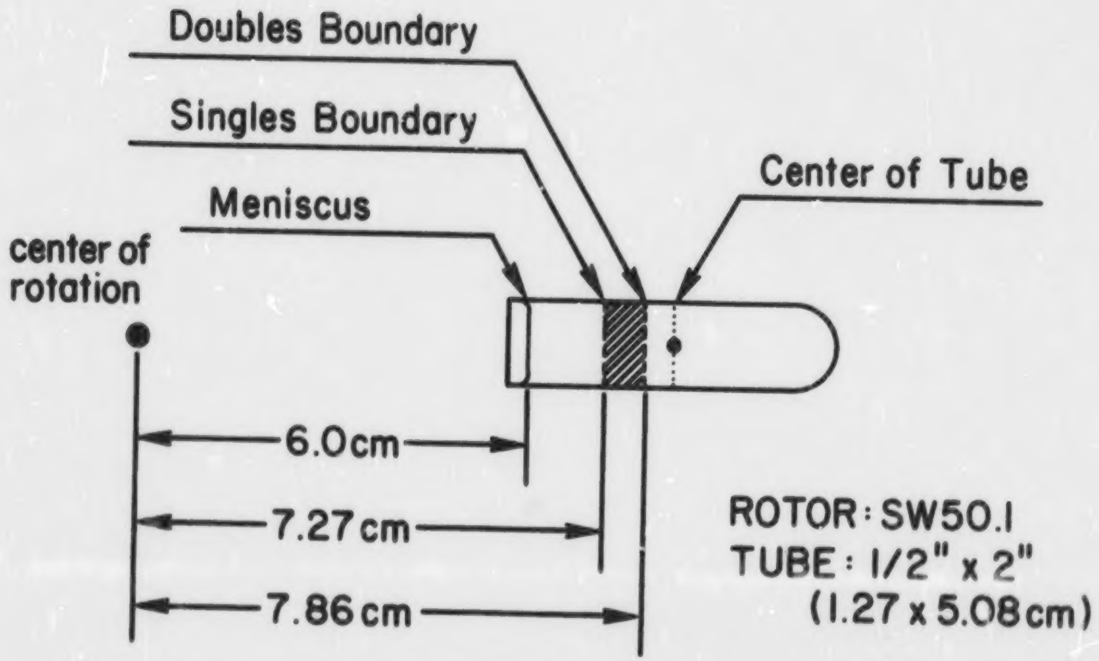

Doubles Boundary
Singles Boundary
Center of Tube
Meniscus
center of rotation
6.0cm
7.27cm
7.86cm
ROTOR: SW50.1
TUBE: 1/2" x 2"
(1.27 x 5.08cm)

Fig. 3 Diagram for locating sample in the centrifuge tube for titration in single particle approximation (SPA) test.

the conditions which place single particles at this level are: rotor speed, 30,000 RPM; time, 20 min.; and temperature, 20C; integrated time at top speed $w^2t = 1.23 \times 10^{10}$ rad^2/sec. Reovirus can be treated in a similar manner with the only exception that the rotor speed is reduced to 15,000 RPM ($w^2t = 3.13 \times 10^9$ rad^2/sec).

A useful technique (24) called the Red, White and Blue (RWB) is an abbreviated rate-zonal experiment that gives an approximation of the percentage of single virions in a preparation as well as a measure of the frequency of small and large aggregates. A small centrifuge tube (5 ml for Beckman SW 50 L rotor) is prepared by placing 1 ml of 50% sucrose solution containing neutral red in the bottom. Two and a half ml of 20% sucrose containing trypan blue are layered over this and 2 ml of the viral suspension to be tested is put on the top. This is spun at a speed and time sufficient to sediment about half of the single virions out of the top (white) section. Preliminary experiments with the best available monodisperse virus showed this to be accomplished in 20 minutes at 30,000 RPM and 20C ($w^2t = 1.28 \times 10^{10}$). As the virions move into the 20% sucrose (blue) section, they sediment slower so that single particles in original 2 ml volume will be compressed into a smaller volume (shaded, Fig. 4).

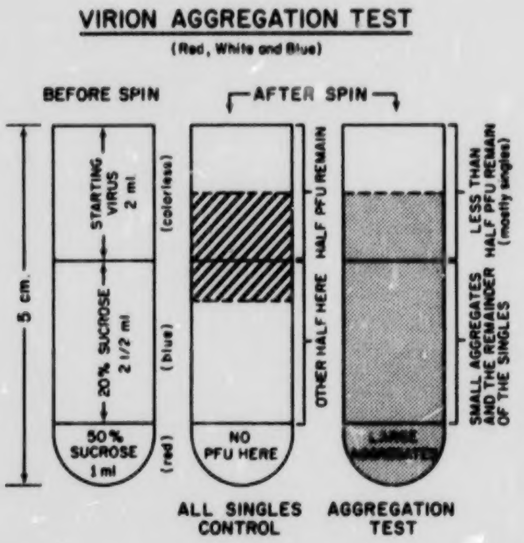

VIRION AGGREGATION TEST
(Red, White and Blue)
BEFORE SPIN
AFTER SPIN
5 cm.
STARTING VIRUS 2 ml
20% SUCROSE 2 1/2 ml
50% SUCROSE 1 ml
(colorless)
(blue)
(red)
NO PFU HERE
HALF PFU REMAIN
OTHER HALF HERE
LARGE AGGREGATES
LESS THAN HALF PFU REMAIN (mostly singles)
SMALL AGGREGATES AND THE REMAINDER OF THE SINGLES
ALL SINGLES CONTROL
AGGREGATION TEST

Fig. 4 Diagram of an abbreviated method for analyzing a suspension of mixed aggregates by separating them into 3 arbitrary parts in a step density gradient for PFU titration. After centrifugation there are mostly single particles remaining in the top (White) section of the 5 ml swinging bucket tube. The middle (Blue) section has the rest of the singles and small clumps while all the large clumps are in the bottom (Red) section.

Pairs, which sediment approximately 40% faster than singles should be 70% removed from the white section and negligible numbers of larger aggregates should remain. So with a monodisperse poliovirus suspension, one should find half of the PFU remaining in the white, the other half in the blue and none in the red section. With polydisperse preparations, we estimate, without experimental evidence at this time, that the largest compact adhering group that could remain in the blue section would be 8 particles. All larger groups would be in the bottom red section. In actual practice better reproducibility in removal of the top (white) section for PFU titration was obtained when a small amount of the blue section was taken with it. In the experiments reported below a total of 2 1/2 ml were taken with the top section and the average of 8 repeat tests with a highly dispersed virus preparation gave 66% (55 to 75%) of the original titer remaining in the white fraction. In these same tests the bottom (red) averaged 3% with a range of 0 to 7%. This might have been a few aggregates or it might have been a small amount of virus left by incomplete removal of the overlaying fractions.

EXPERIMENTS AND RESULTS

STABILITY OF REOVIRUS SUSPENSIONS

Virus taken from the prominent band formed in the sucrose gradient was pelleted twice to remove sucrose and suspended in 80 ml of 0.005 M phosphate buffer at pH 7.0. Five electron microscope (EM) pictures were taken at random on agar sedimentation preparations made immediately. The number of particles per picture was 173, $\sigma = 25$, and the $\log_{10}$ of the total number of singles, doubles, triples, etc., is shown plotted against $\log_{10}$ group size in the lowest of the three lines of Fig. 5. The straightness and slope of this plot was examined again more critically after the suspension had stood 4 days at 4 to 6 C. This time 25 random pictures were taken, and the average total count per picture was 175, $\sigma = 22$. The log-log frequency distribution of this series is shown in the top line of Fig. 5. The middle line of the figure came from 10 EM pictures taken 21 days after the first set. The total count was 178, $\sigma = 21$. Less complete data of the same kind show that the number and frequency distribution of aggregates is equally stable in 0.05 M phosphate buffer at the same pH. Apparently the degree of aggregation that existed when the virus was first resuspended from the last pellet was not altered in 21 days, and no significant loss of virus to the glass walls of the storage bottle was noted even though no special precautions were taken to prevent it.

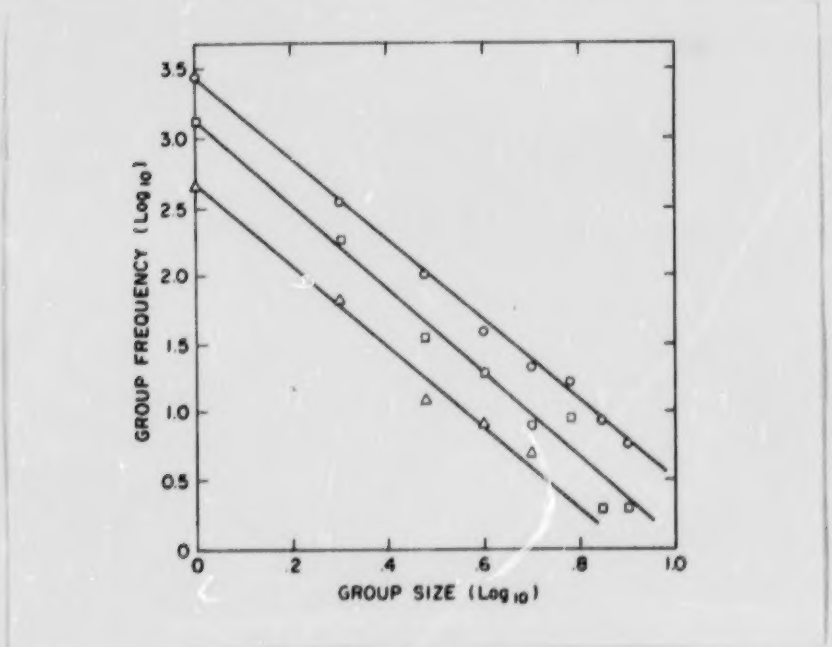
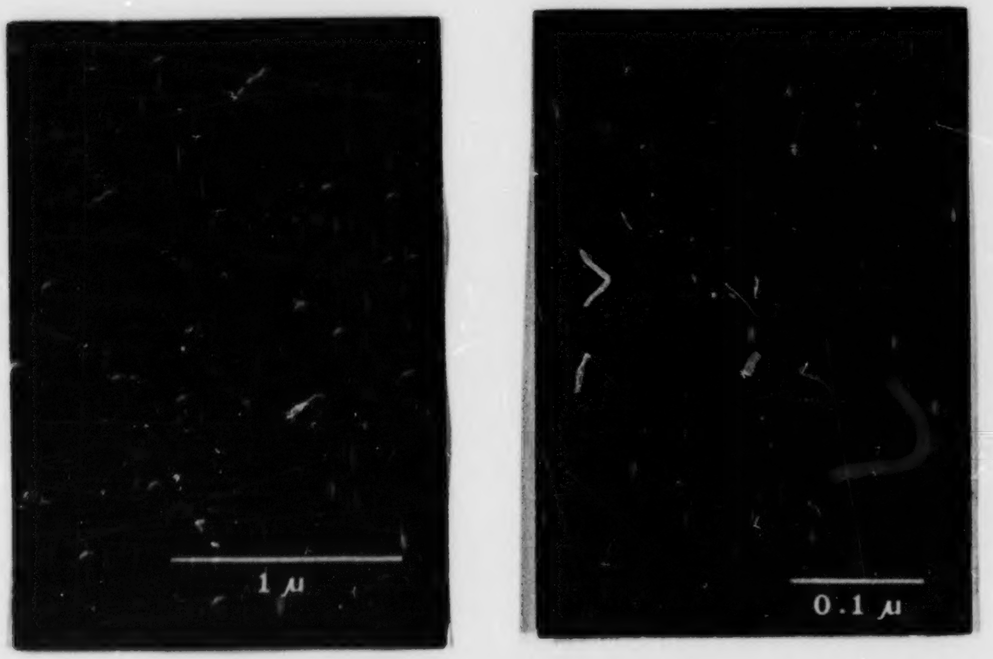


Fig. 5 The state of small-number aggregates of 10^8 reovirons/ml of 0.005M phosphate buffer (pH7) is shown in terms of group size and frequency. Data are from electron micrographs made immediately (Δ), after 4 days (o) and after 3 weeks of storage at 4 to 6 C (□).

The effect of dilution on the state of aggregation was observed using virus as a concentration of 3 x 10^9 per ml. This count was verified and the frequency distribution of aggregates was measured in EM pictures made by the kinetic attachment method. These virions became attached to the collodion film during Brownian motion in this suspension at high concentration. Then this suspension was diluted 50 x with 0.005 M buffer, and another set of EM pictures was made by the agar sedimentation method. Approximately the same number of virions was present on both sets of pictures, and the log-log plots of group frequencies were as near alike in slope as those of Fig. 5. Thus, within the limits available for direct observation of reovirus particle aggregation in 0.005 M phosphate buffer at pH 7, the state of dispersion is independent of virion concentration.

YIELD AND PHYSICAL APPEARANCE OF THE REOVIRUS. When Freon-extracted virus from about 80 million L cells was velocity-banded on the sucrose gradient, about 5 x 10^{11} virions were regularly recovered from the prominent, rapidly moving band. These are shown shadowcast and negatively stained in Fig. 6a and 6b, respectively. In a faint,



Figs. 6a and 6b Fresh reovirus (untreated) shadowcast (a) and negatively stained (b).

①

slower sedimenting band, as many as 1.3 x 10^{11} particles have been recovered. These "top component" (25) particles are shown in Fig 7. Many of them are flattened in the shadowcast pictures Fig 7a and appear empty in the negatively stained preparations Fig 7b. These pictures are shown here for comparison with the bromine-treated virus below.

1/40

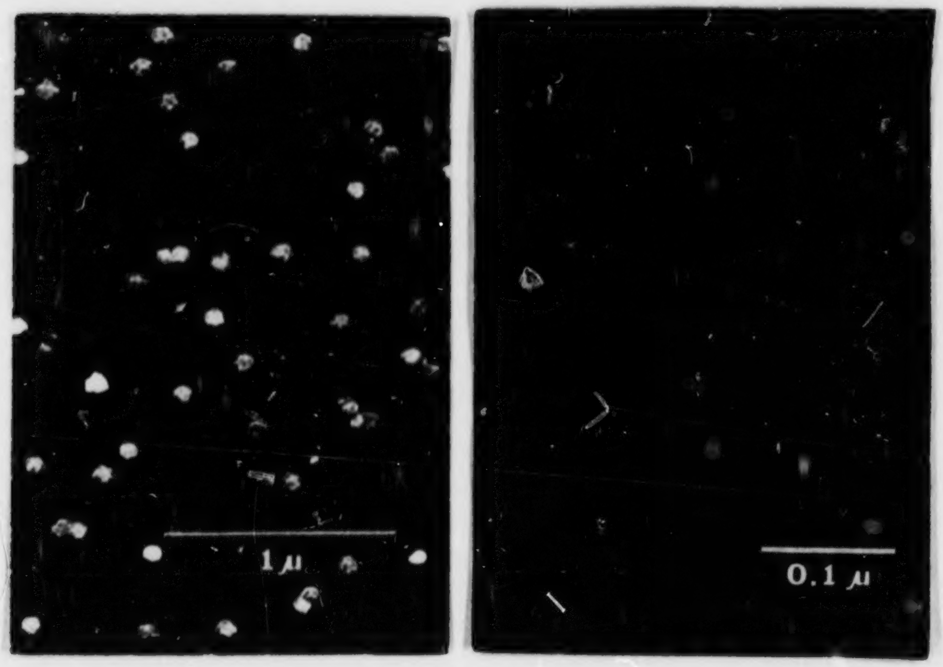


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Fig. 7 Virus-like particles from the slower sedimenting "top component" shadowcast (a) and negatively stained (b) are usually hollow in the center and their shadows are usually shorter, indicating defective structure.

2

EFFECTS OF BROMINE (HOBr) ON THE REOVIRION. The survival ratio of PFU is shown plotted as a logarithm against the time of treatment for several concentrations of bromine as HOBr (Fig. 8). The virion concentration was in the range of 10^8 to 5×10^8 particles/ml, and in 12 such experiments the degree of aggregation among the in-going virions ranged from a slope of -1.78 to -3.12 on the log-log plot of frequency versus group size for groups of size from 1 to 10. In spite of this substantial amount of aggregation, the reaction rates were too fast for accurate measurement during the initial stages even at HOBr concentrations as low as 3 μM (0.48 mg Br_2/l). Below this value it was difficult to maintain a constant bromine concentration. In the experiments reported here the bromine concentration from the beginning of the disinfection run never varied by more than 1 μM (0.16 mg Br_2/l). Nevertheless, all the lines were curved. Curvature was usually detectable at the 10^{-2} survival level but always before the 10^{-4} level was reached, indicating that the resistance of surviving PFUs was continuously increasing. This effect might have

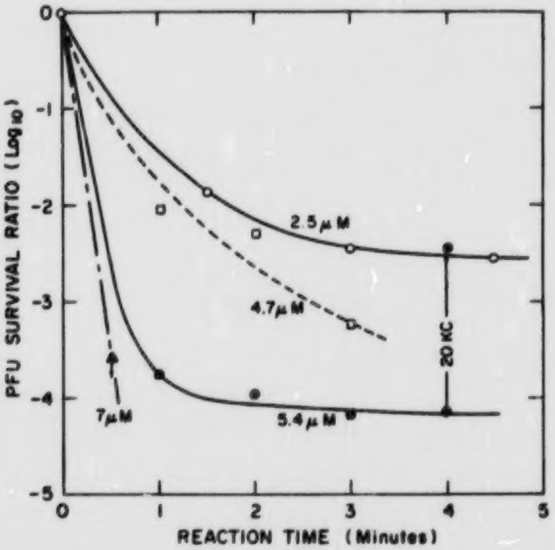


Fig. 8 Loss of PFU titer of reovirus in water containing bromine (HOBr).

been due to depletion of the free HOBr during the reaction, but experiment (Fig. 9) shows that, after the reaction had essentially stopped, fresh virus added to the same mixture was destroyed at a rate no less than that of the original inoculum.

Virus recovered from the reaction mixture was diluted 1:1 in the neutralization by sodium thiosulfate, but its concentration was still high enough for count and aggregation analysis by the agar sedimentation process of preparation for electron microscopy. The virions were found in the expected number, and very little additional aggregation was

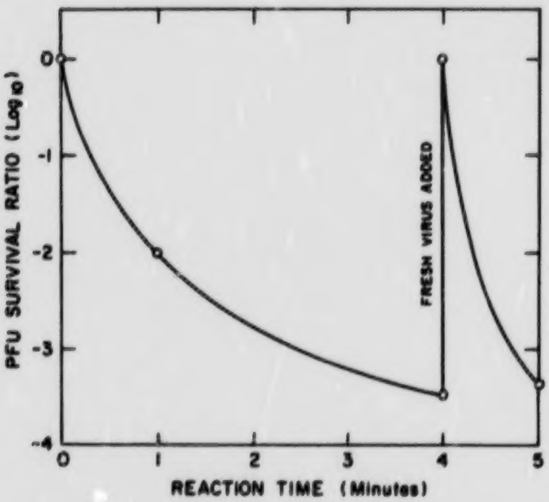


Fig. 9 Typical bromine inactivation experiment showing that the rate of decline in reovirus titer is not due to depletion of the active agent; fresh virus, added after 4 min, was inactivated at a high rate.

induced either in the brominated mixture or by the sodium thiosulphate. The conspicuous observation, made on all the bromine experiments, was the change in appearance of the virions (Fig. 10). They lost a large fraction of their substance, including enough structural elements so that the residue made a very flat image (Fig 10a), much more so than even the imperfect particles in the normal "top component" of Fig. 7 and many of the virions looked ragged and empty when negatively stained (Fig. 10b). Still, the number of these residues was the same as the number of virions put into the mixture. Apparently very few or none disintegrated beyond recognition in the electron microscope.

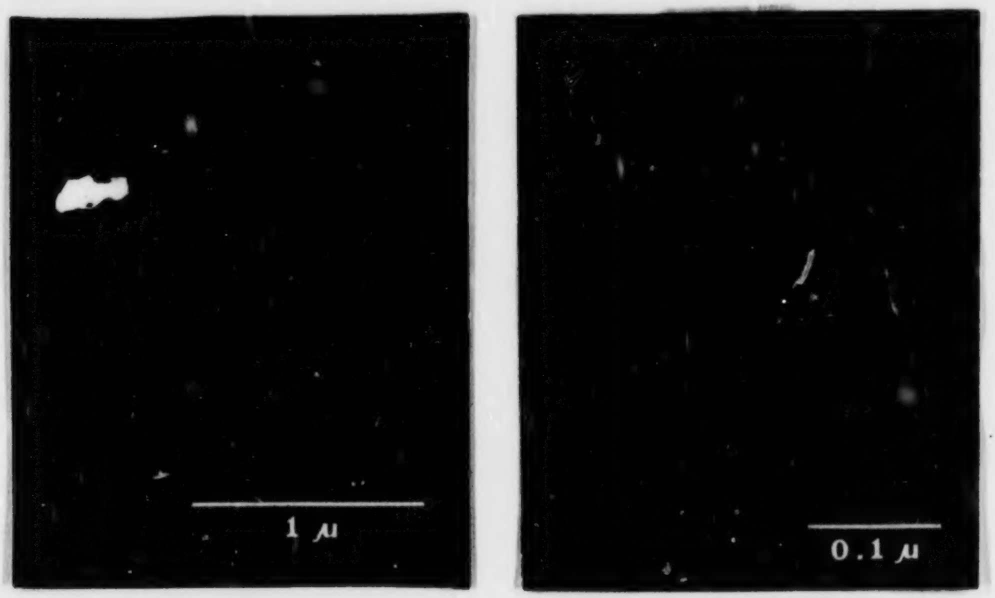


Fig. 10a and b Reovirus, after treatment with bromine, has lost both substance and structural form as shown here shadowcast (a) and negatively stained (b), but the number of these heavily damaged particles remains the same. No detectable fraction of the input particle count is lost.

⑬ 14X

DISPERSIVE TREATMENT OF BROMINE-TREATED VIRUS. When 20-kHz waves were applied after neutralization of the bromine, the surviving plaque titer increased 10-fold in two experiments and 43-fold in a third (Fig. 8). No significant change was observed in the number of aggregates in the size range 2 to 10, but this was difficult to observe accurately with the badly damaged particles. Still, the virions did not disintegrate further, for their numbers did not decline. This led to a search for a few PFU that were substantially larger than either single virions or small clumps.

PHYSICAL NATURE OF SURVIVING PFU Virus was treated with 5 μM (0.80mg $Br_2/1$) HOBr for 4 min. The neutralized mixture (5 ml) was placed in a single tube of a Beckman SW50 rotor and centrifuged at 40,000 rpm for 30 min at 5 C. The total integrated effect of this procedure was equivalent to 33 min at 40,000 rpm, and this would have been quite sufficient to sediment all single virions and clumps to the bottom of the tube. Less than 1% of the surviving PFU were found in the supernatant fluid after this centrifugation. Certainly there were no significant numbers of plaques produced by ribonucleic acid released from the virions by the HOBr.

At this point it seemed unlikely that aggregates sufficiently large to provide protection for even one virion could have accumulated soon enough after the virus was added to the HOBr solution. Therefore, the next experiment sought to remove hypothetical aggregates, too few to be noticed by electron microscopy but presumably present before HOBr treatment. Inasmuch as at least 16 spheres are required to form a protective coat one sphere thick around one of equal diameter, and such a clump must have a radius about three times that of one of the spheres, we chose first to remove them by centrifugation at a speed of 11,300 rpm in an SW50 rotor for 18 min. A 5 ml amount of the starting virus was subjected to this treatment, and 4 ml of the supernatant fluid was carefully removed for HOBr treatment done in parallel with uncentrifuged starting virus. EM counts showed that the number of virions per milliliter of the supernatant fluid used was not significantly less than that of the control. After treatment of both with 3.3 μM (0.52 mg $Br_2/1$) bromine for 1 min, the control virus produced $10^{3.23}$ plaques/ml, whereas the centrifuged virus produced less than $10^{1.3}$plaques/ml. Essentially the same result was also achieved by treating the starting virus preparation with 20 kHz waves prior to bromine treatment. Apparently the potential surviving PFU are large clumps which exist before bromine treatment. They are few enough to escape detection when only a few hundred virions are observed on EM pictures used for routine counting.

Isopycnic banding of virus after 4 min exposure to 5.4 μM (0.84mg Br_2/l) bromine was done by adding CsCl crystals to the reaction mixture after neutralization with sodium thiosulfate. One tube of the SW50 (Beckman) rotor was filled with this homogenous virus suspension at a starting density of 1.22 g/ml and spun at 35,000 rpm for 20 h at 5C. The virus was located by EM count in a single peak at $\rho = 1.25$ g/ml (Fig. 11).

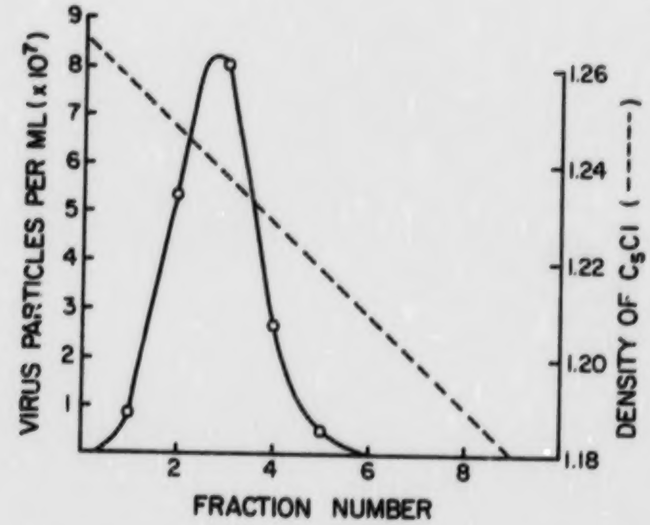


Fig. 11 Bromine-treated reovirus forms this isopycnic band at a CsCl density of 1.23. Fresh reovirus bands at a density of 1.36 (9).

Velocity banding was tried by placing 10 ml of bromine-treated reovirus over a 20 to 40% (wt/wt) sucrose gradient in an SW27 Beckman centrifuge tube and spinning at such time and speed that single virions might move appreciably into the sucrose before PFU were all sedimented to the bottom. Even with the established isopycnic density of the bromine-treated virus, we were not able to predict nor even measure accurately the relative sedimentation velocities because of the difficulty of precisely locating the starting position of the particles. Nevertheless, the experiment did clearly establish the fact that the virus particle peak located by electron microscopy contained no PFU, and no particles (or clumps) could be found by electron microscopy in the region of the much more rapidly sedimenting PFU peak (Fig. 12). Although the two peaks are drawn (for convenience) with approximately equal area, the number of virions per milliliter and the number of PFU per milliliter in the respective peak regions are actually in the ratio of about 1,000,000 to one.

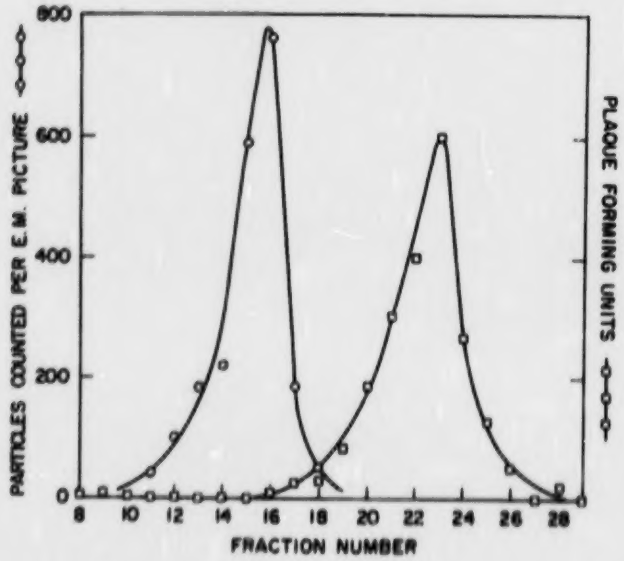


Fig. 12 Surviving reovirus PFU are aggregates, not single particles.

Bromine-resistant virus has not yet been found except in clumps. Virus populations, grown from plaques isolated at the 10^{-4} survival level in bromine experiments, have shown the same sensitivity to bromine as the original population.

THE INITIAL FAST REACTION OF HOBr WITH REOVIRUS. One of the results described above showed the need for critical examination of reaction kinetics at contact times much shorter than one minute. For this work the Dynamic Inactivation apparatus was used. Several experiments were made at 10 C with samples taken at 4 sec intervals. These were made with virus prepared as previously described (3), which involved pelleting of the purified virus to remove sucrose. These were done at 3, 5.8, and 5.9 mM (0.48, 0.73 and 0.95 mg Br_2/1) HOBr concentrations (Fig. 13), and they all show approximately the same level of persistent resistance previously reported in exposures of longer duration. They show also that the initial fast reaction is taking place during the first 4 sec interval. Virus was titrated (upper dotted line) after passing through the apparatus in exactly the same way, without any bromine in the flowing stream. Virus dilution was equal to the ratio of stream velocity (39.6 ml/sec) to injection velocity (1.11 ml/sec) times 2 for the dilution with the sodium thiosulfate (2 x 39.6/1.11 = 71).

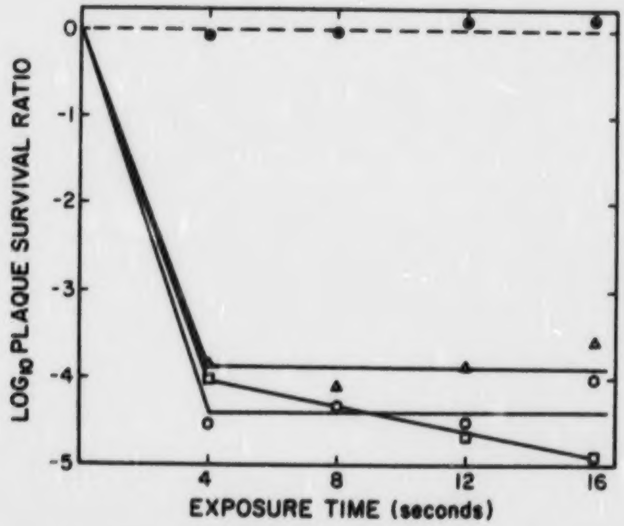


Fig. 13 Persistent survival of large aggregates of reovirus during bromine treatment.

Each titer was multiplied by this factor and then divided by the titer of the starting virus, and the logs of these ratios are plotted as filled circles at the top of Fig. 13. They show, by their small deviation from the dotted horizontal line drawn through them, that no changes in plaque titer occur as the control virus passes through the apparatus, except those due to dilution.

At this po

At this point we added the magnetic mixing devices to the sampling syringes and reduced the length of the flow tube to give samples at 1 sec and later at 0.5 sec intervals. The temperature was reduced also, from 10 to 2C, to reduce the reaction rate and still permit the use of bromine concentrations high enough to hold constant when confronted with expected bromine demand of the virus.

Three experiments were made at 2.8 to 3.0 μM (0.45 to 0.48 mg Br_2ll) HOBr concentration with virus prepared without pelleting. Two were done with 1 sec time intervals and one with the intervals reduced to 0.5 sec. The frequency of aggregates observed by electron microscopy in this virus preparation is shown in Fig. 14.

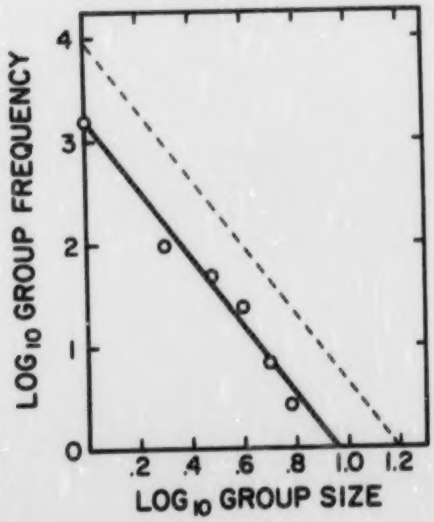


Fig. 14 Frequency distribution of aggregates observed by electron microscopy (circles). The dotted line indicates the same distribution for a more concentrated suspension containing a total of 10,000 particles.

Of the whole particle population, 73% are singles, and the groups appear to be distributed in such a way that the log frequency of each group size is a linear function of the log of the number in that group. We (26) and others (27) have observed this same distribution with other viruses and also with polystyrene and acryl particles of comparable size. The inactivation of the virus by HOBr in these three experiments is shown by the open circles, triangles and squares in the semilog plot of survival ratio versus time in Fig. 15. It is curved all the way. A single line has been drawn by inspection through all three sets of points. Correlation of this result with the aggregation distribution (Fig. 14) will be made later (see Discussion).

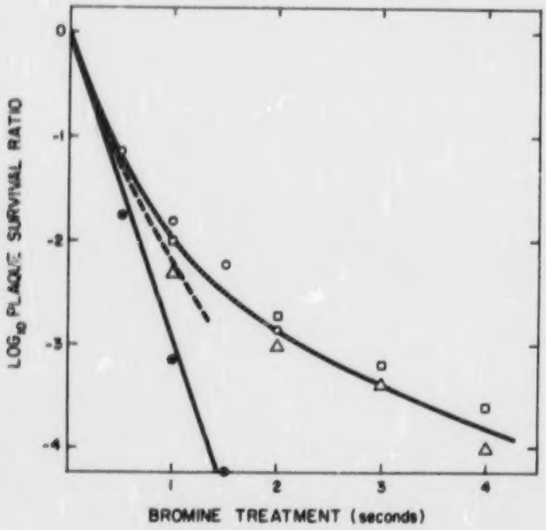


Fig. 15 Inactivation of reovirus during the first 4 s of contact with 2.9 μ M bromine in water at pH 7 and 2 C. Three separate experiments are shown with different symbols. The frequency of aggregates of different sizes is that shown in Fig. 14. Filled circles show the fast inactivation rate, where the suspension contains essentially all single particles. Dotted line shows survival calculated from the observed frequency of aggregates and the Poisson limit as described in Discussion.

Zonal Centrifuge Experiment ZR1. An effort was made to obtain a preparation of virus containing only single particles. This is needed to provide a frame of reference for comparison with the above results with aggregated virus. A 15-ml amount of a purified virus preparation, aggregated as shown in Fig. 14, was layered over a 15 to 30% (wt/wt) sucrose gradient with the BXIV zonal centrifuge rotor running at 20,000 rpm. The total number of virions put into the rotor was 10^{11} by electron microscope count. After operating at 25,000 rpm for 30 min ($\Sigma\ \omega^2 t = 10^{10}$) at 20 C, the 640-ml volume of the rotor was completely displaced by 35% sucrose piston fluid, and 25-ml fractions were collected in 1-ounce bottles. The virus particle count on each was made by electron microscopy after dilution with 0.85% filtered sodium chloride solution. A dilution of at least 1/10 is required; otherwise the agar block that receives the sedimented virions in the pseudoreplica counting process will float to the surface because of residual sucrose. Particle counts are plotted against fraction numbers, and a scale of radial distances is included on Fig. 15. Densities in the sucrose gradient were measured by direct pyknometer weighings.

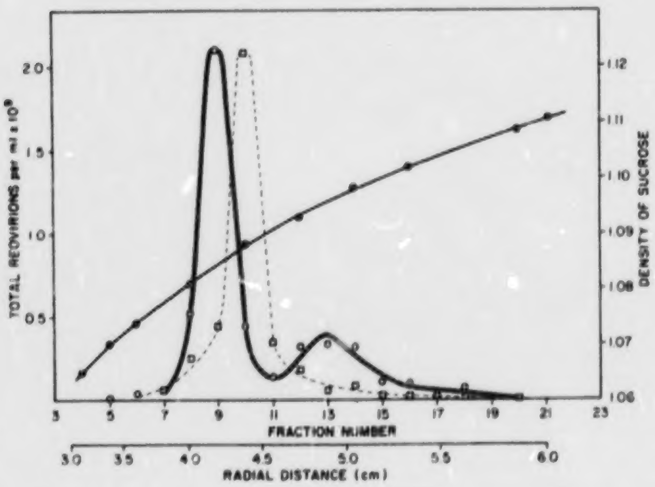


Fig. 16 Sedimentation velocity analysis of reovirus in a zonal centrifuge rotor. Exp 1, heavy solid line, Exp 3, dotted line. Sucrose density is shown by the light solid line.

Fractions 4 through 10 contained 69% of the recovered virus. In the peak fraction (no. 9), there were 93% single particles. Six percent of the remainder was in pairs, and the remaining 1% was in triplets and groups of four (Fig. 17, bottom line). Thirty-one percent of the total recovered virus made a broad secondary peak with maximum count in fraction no. 13, in which 63% of the particles it contained were in pairs. The total count of all the fractions indicated 110% of the 10^{11} particles that were put in. Apparently there was no detectable loss in the partition process, and calculations from the electron microscope frequency distribution chart (Fig. 17 upper line) predict that 71% of the in-going population was single particles, a figure in excellent agreement with that observed in this actual sedimentation velocity spectrum from the zonal centrifuge.

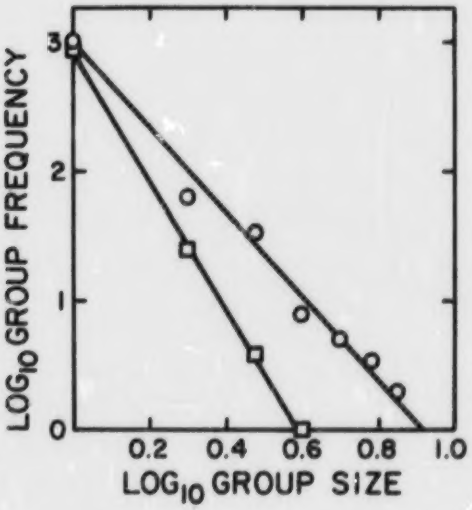


Fig. 17 Frequency of aggregates observed in the starting virus preparation used for Exp 1, Fig. 16 (circles) and in the peak region, fraction 9 (squares)

Zonal Centrifuge Experiment (ZR2). Inasmuch as zonal selection provides a method of obtaining a very high percentage of single reovirus particle suspensions, we prepared a fresh quantity of purified virus containing about 50 times more virus than that of the above zonal fractionation. This preparation yielded the velocity spectrum with single peaks like that of Fig. 16 but fractions 12 through 19, which contained aggregates of two to eight particles constituting 31% of the first population examined, now contained aggregates in the same size range but relatively few of them (less than 10% of the total). Apparently, freshly prepared Freon-extracted virus has very few aggregated particles. In the peak fraction (no. 10) there were 87% singles. It is possible that at this high concentration (2.5×10^{10} virions/ml) some spontaneous aggregation took place before preparations could be made for electron microscopy. A part of this fraction was frozen immediately to preserve both infectivity and physical dispersion for subsequent bromine inactivation experiments.

Zonal Centrifuge Experiment (ZR3). One more zonal velocity spectrum experiment was made with a crude freeze-thaw lysate of infected L cells without even low-speed clarification. This crude preparation contained the virus from the same number of infected cells as that providing virus for the last experiment, described above. Again a single particle peak was observed (Fig. 16), and again there was very little virus in the region in which aggregates of two to eight were seen in substantial numbers in the first experiment. This unexpected result drew attention to the fact that the total quantity of virus recovered from the crude starting material was only about 1/5 of that usually recovered by Freon extraction of an equal number of infected cells. It would appear that an excellent fraction of single particles can be obtained in this way with no previous purification and that there are very few small aggregates in such a preparation, but that the major part of the virus must have been in large aggregates or combined with larger cell debris which sedimented beyond the sampling range and reached the rim of the zonal rotor.

Bromine Inactivation of "Singles" Fraction from Velocity Spectrum. The singles peak fraction from zonal velocity spectrum (ZR2 above was frozen at -40 C in 1-ml vials to preserve infectivity and state of dispersion. Subsequently thawed samples had not lost infectivity, and electron microscopy did not show any change in the relative numbers of single particles and groups in the size range of two to eight. But bromine inactivation showed that changes had nevertheless taken place. After a rapid start, the reaction became slower and appeared to reach a resistant level at about 10^{-3} survival after 2 sec at 3 μM (0.48mg Br_2/l) HOBr and 2 C. If these few surviving PFU are aggregates, they must have "grown" from single particles during the short time interval before freezing or in the thawing process.

Five milliliters of the same virus were treated with bromine in the same way, except that they were centrifuged for 17 min at 20,000 rpm at 20 C in a Beckman SW50 rotor. Only the top 4 ml were used in the bromine experiment. All large aggregates should have been removed from this preparation. The resulting rapid linear decline in PFU (Fig. 15, solid points) provides excellent confirmation.

Partition of Crude Reovirus Between Singles and Large Aggregates by Means of a Sucrose Gradient in the Swinging Bucket Centrifuge Rotor. Zonal sedimentation velocity spectra of crude reovirus (ZR3 above) yields a prominent sharp band of single virions and relatively few small aggregates, but most of the virus seems to have sedimented much faster than either. An experiment was done in the large (37ml) bucket of the Beckman SW27 centrifuge rotor to determine, if possible, the quantity and physical state of virions and the plaque titer of the two major components of this particle population, the singles band, and the pelleted material. To do this, a 20 to 40% sucrose gradient was established and 10 ml of the freeze-thaw lysate (the same as that used in experiment ZR3) was layered over it. After spinning at 25,000 rpm (mean centrifugal field, 81,000 x g) for 1 h, the supernatant fluid above the visible singles band was discarded. A 10ml amount, including the singles band, was collected, and then the pelleted material was resuspended in the remainder of the supernatant fluid by pumping with a pipette. These two fractions will be called band 1 (B1) and pellet 1 (P1).

An equal quantity of the crude virus preparation was extracted with Freon and layered over a similar sucrose gradient, centrifuged, and harvested in the same manner, band 2 and pellet 2.

One-half of the resuspended pellet 1 material was extracted with Freon, made up to the same volume (10 ml), and banded and harvested like the previous ones, band 3 and pellet 3.

A part of both fractions B1 and P1 were treated for 30 sec with 20-kHz acoustic waves from the microtip of a Branson Sonofier model LS-75. Sample volumes treated were 5 ml each, and they were immersed in ice water during treatment. The maximum temperature attained was 15 C. There were eight samples in all prepared for virion count and observation of aggregation in the electron microscope and for plaque titration. The results of physical assay are shown in Fig. 18. Particle count of pelleted fractions from Freon-extracted preparations contained a large amount of cellular debris, which made the virions difficult to count. Nevertheless, the total particle yield (sum of counts from pellet and band) was approximately the same (Fig. 18). Freon extraction yielded 57% of the particles in the band, but with the crude virus only 28% was in the band. Freon extraction of virus from the pellet fraction of the crude virus experiment (P1) yielded 67% of the particles in the band. Apparently the Freon extraction process is quite efficient for extraction of reovirus in monodisperse form from the infected cells.

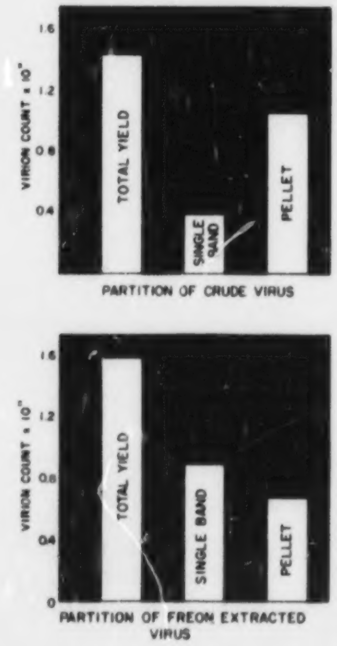


Fig. 18. Partition of virus between singles and aggregates.

Plaque titration of banded and pelleted fractions from several identical partition experiments have shown 1/7 of the Freon-extracted virus to be in the pellet and 6/7 in the band. The partition of PFU in crude preparations has been erratic. The pellet fraction always has much more than the band, and once there were 50 times as many PFU in the pellet as there were in the band. Treatment of banded and pelleted fractions with 20-kHz acoustic waves have not made any substantial change in particle count, but in this case it reduced the plaque titer of the pelleted fraction by a factor of 10. The same treatment made no change in the titer of the well-dispersed virus in the banded fraction.

BROMINE (HOBr) INACTIVATION OF SINGLE POLIOVIRUS PARTICLES.

The dynamic experiment, having been successful with reovirus, was immediately applied to polio. When the virus in these experiments is extracted from the infected HEp-2 cells with Freon and velocity banded in a sucrose gradient, the particle content of several successive fractions from such a gradient is as shown in Fig 19. The concentration of particles calculated from counts from such pictures is plotted against radial position in Fig. 20. Many particles smaller than virus, probably ribosomes, can be seen in fractions 11 and 12 just above the virus peak. These were not counted. At no point below the main virus peak was there any secondary peak in the region where pairs, triplets, etc. would have been expected had they been present in substantial numbers. It appears from this that the Freon extraction process has yielded a virus preparation with a very high proportion of single particles.

The strip pictures of Fig 19 were taken at high particle concentration, presenting enough particles so the reader may judge their relative number and degree of purity. Other pictures made with more dilute preparations were used to determine the true degree of aggregation, free of the effect of one particle falling upon another by accident. Calculation of the number of such accidental pairs will be made later (see Discussion). They indicate that the starting virus for these experiments contains at least 95% single particles.

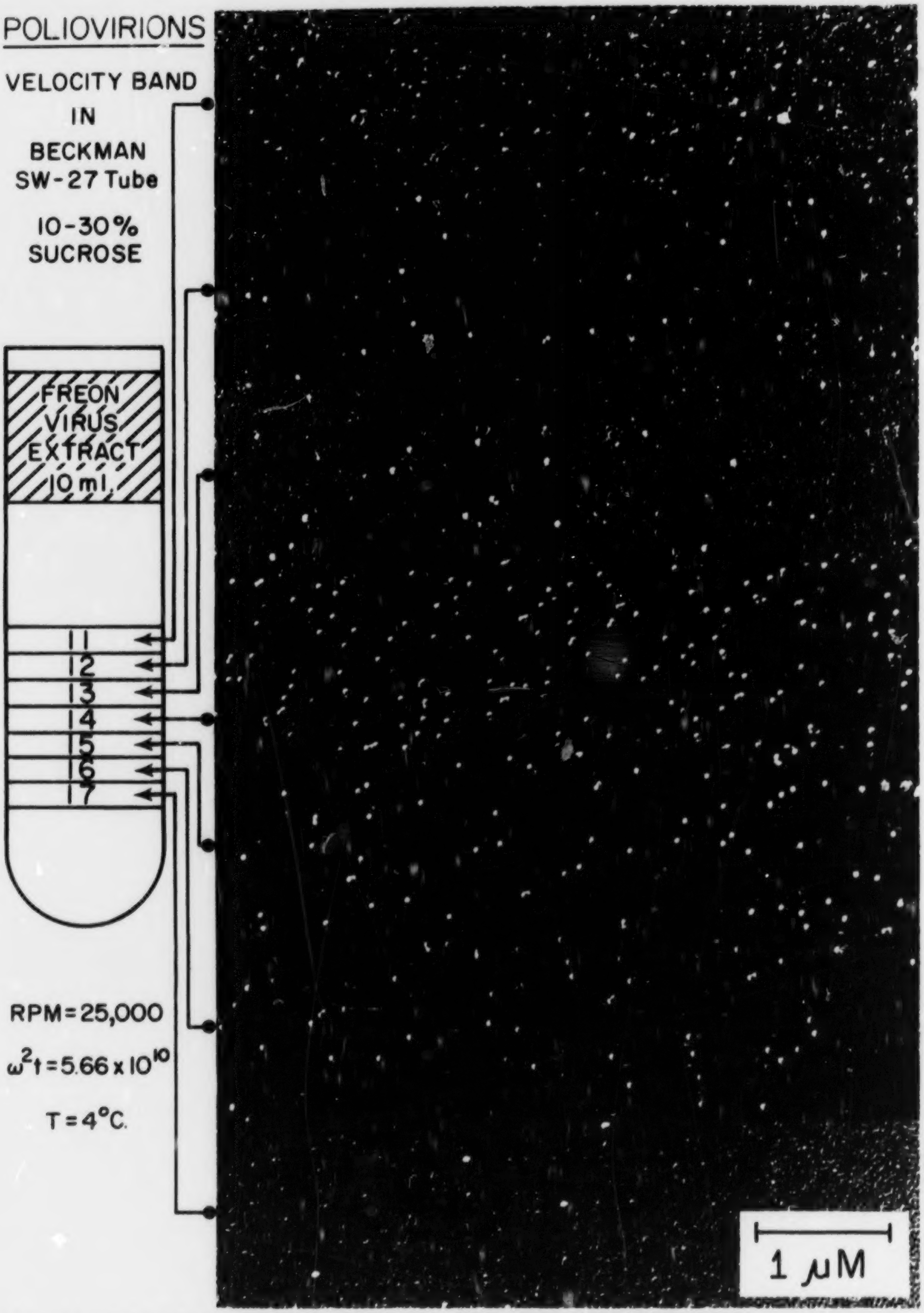

POLIOVIRIONS
VELOCITY BAND
IN
BECKMAN
SW-27 Tube
10-30%
SUCROSE
FREON
VIRUS
EXTRACT
10 ml.
1
2
3
4
5
6
7
RPM=25,000
ω²t=5.66x10¹⁰
T=4°C.
1 μM

Fig. 19 Poliovirus, velocity-banded in a sucrose density gradient.

Move ^

(15) 16X

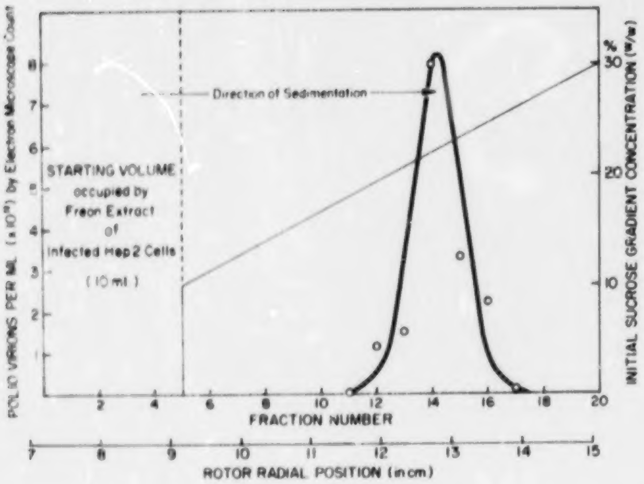


Fig. 20 Graphical display of poliovirus particle count by electron microscopy, taken from the fractions and pictures shown in Fig. 19.

Poliovirus Inactivation by Bromine (HOBr) at 2 C and pH 7. Six experiments were made at 2 C, three of which are shown graphically in Fig 21. All were characterized by an initial linear phase and constant decline in log plaque-forming unit survivor ratio per unit time of exposure. None of these reactions showed any tendency toward delay during the first time interval, which was 4 sec. The slopes of all the reactions, ranging from 0.6 to 22 μM (0.01 to 3.5 mg Br_2/1) concentration, have been plotted in Fig 22, where it can be seen that increasing bromine concentration does not produce a proportionate increase in reaction rate. The increase becomes progressively less with increasing bromine concentration, indicating a progressive decrease in efficiency.

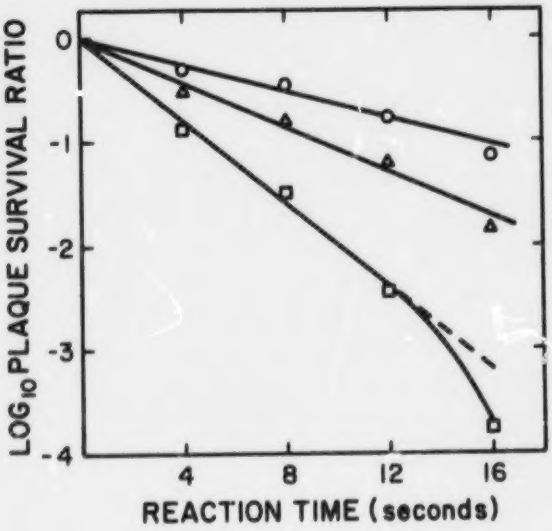


Fig. 21 Inactivation of poliovirus at three different concentrations of bromine at pH 7 and 2 C: (○) 0.6 μM; (△) 2.2 μM; (□) 22 μM.

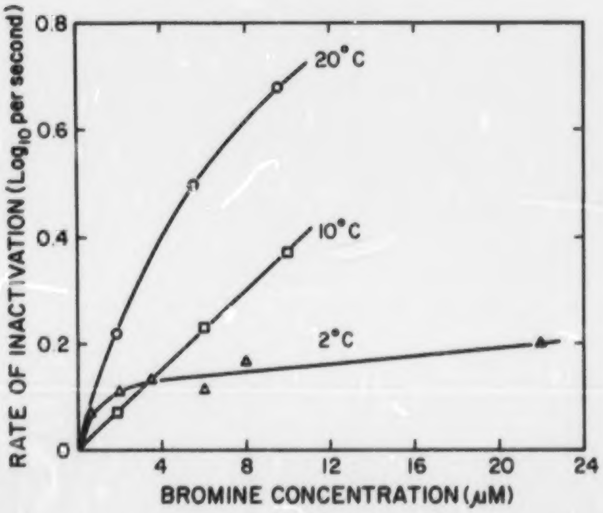


Fig. 22 Inactivation rates (slopes in logs per second taken for Fig. 21,23, and 24) for poliovirus at pH7 as function of temperature and bromine concentration.

In several inactivation experiments at HOBr concentration of 3.5 μM (0.56mg Br$_2$11) or greater, there has been a slight increase in the reaction rate after a substantial period of linearity (see the 22 μM (3.5mg Br$_2$11) line in Fig 21). We have not been able to observe this effect regularly. Slopes for Fig 22 were taken from the linear part of each survival graph.

Poliovirus Inactivation by Bromine (HOBr) at 10 C and pH 7. At 10 C the inactivation rate was constant at 1.9 μM (0.30 mg Br$_2$11) HOBr concentration (Fig 23). There is no indication, in this experiment, that there is any time delay before inactivation begins. However, at higher bromine concentrations the linear part of the graph begins only after the first 4 sec time interval (Fig. 23). It seems unlikely that the initial rate at the higher concentrations could be less than that of the straight line for 1.9 μM (0.30mg Br$_2$11) HOBr, so the lower curves have been drawn tangent to it at zero time. The reaction rates, slopes of the linear parts of the three kinetic experiments, are shown (Fig. 22) to be a linear function of bromine concentration, with an intercept indicating zero reaction for zero bromine.

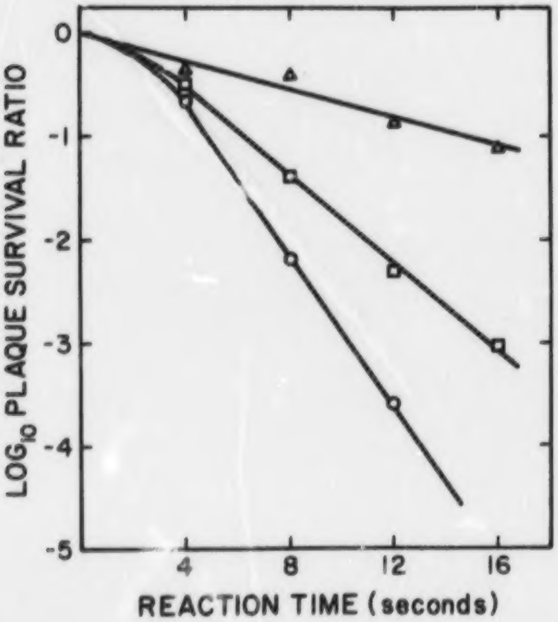


Fig. 23 Inactivation of poliovirus at three concentrations of bromine at pH7 and 10 C: (△-△) 1.9 μM; (□-□) 5.9 μM; (○-○) 10 μM.

Poliovirus Inactivation by Bromine (HOBr) at 20 C and pH 7. At 20 C the inactivation of the virus proceeds faster at all bromine concentrations than it did at 10 C (Fig. 24). There is some indication in the graph for the 9.5 μM (1.5mg Br_2/1) HOBr experiment that aggregation is showing its effect at several levels below 10^{-3}. Reaction rates were determined from the straight part of each line.

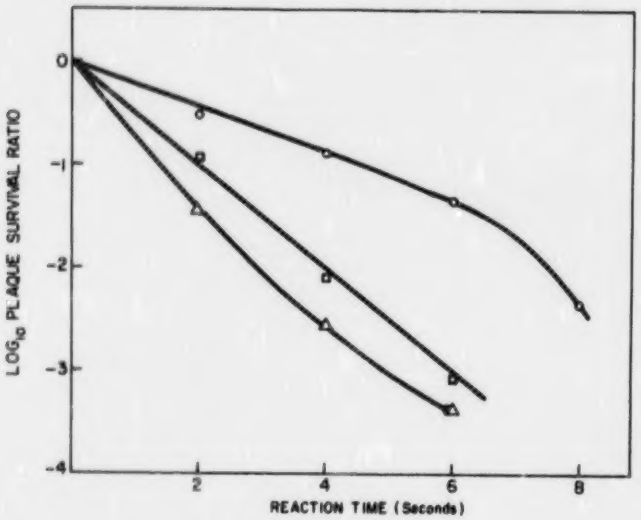


Fig. 24 Inactivation of poliovirus at three concentrations of bromine at pH and 20C: (○) 1.9 μM; (□) 5.5 μM; (△) 9.5 μM.

AGGREGATION OF POLIOVIRUS AND REOVIRUS BY DILUTION IN WATER (29)

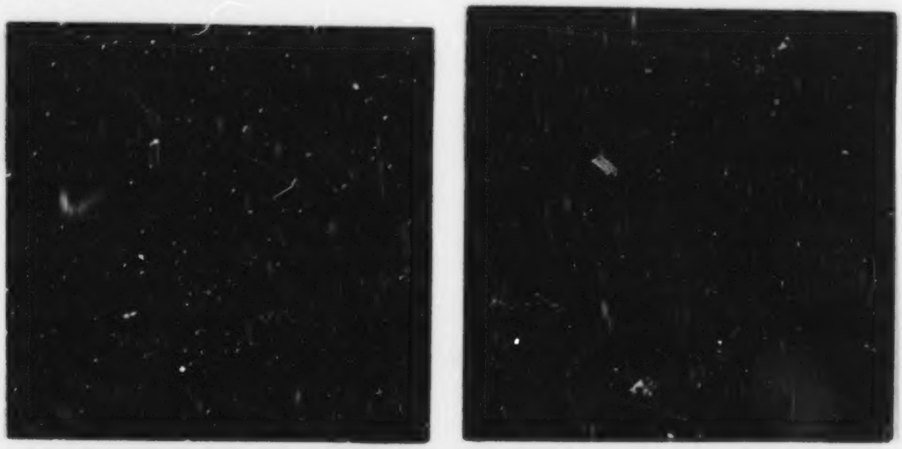
The influence of virion aggregation on the survival of infectivity in water containing halogens and their compounds has long been suspected (1). Direct observation of the virion aggregation in disinfection experiments with bromine has been made recently with both poliovirus and reovirus (3, 5, 28), in which it was found that aggregated virus was substantially more resistant than suspensions of single particles. This resistance was, in one case with reovirus (3), shown to be due to the presence of large aggregates exerting a protective effect on interior particles. Other effects of aggregation such as complementation and/or multiplicity reactivation could also conceivably occur with small aggregates after inactivation by bromine, chlorine, or other compounds. Although this possibility has not yet been rigidly documented, it has been suggested in an earlier paper (5).

Ideally, any comparison of the relative resistance of two different viruses to one disinfectant must be made first on suspensions of single particles. However, the aggregated state is difficult to avoid in the laboratory, and certain precautions must be taken to insure a monodispersed suspension of virus. Animal viruses are usually gathered and maintained in salt solutions chosen for best maintenance of infectivity. Optimum ion kinds and concentrations, usually similar for different viruses, and ranges of pH stability have been established for different viruses. On the other hand, drinking water, and the lake and river waters that are the usual sources for drinking water, do not provide the optimum ions that are used in the laboratory. Therefore, it is reasonable to expect that viruses may not react with predictable behavior in raw or finished water, or in water passing through sewage or water treatment plants. This paper establishes, by electron microscopy and sedimentation velocity, some of the aggregation changes that two common laboratory viruses undergo when placed in water and under other non-physiological conditions.

Poliovirus. Electron Microscopy. The examination of virus aggregation requires a stock virus preparation in a well-defined monodispersed state. The poliovirus stocks used in this work were prepared by Freon extraction followed by sucrose density gradient sedimentation in 0.05 M phosphate buffer, pH 7.2 (28). The virus band, collected from the gradient, was stored at 4C without removal of the sucrose, the concentration of which was approximately 22%. The particle count of these preparations was 7×10^{11} to 2×10^{12} particles/ml in the dispersed state. Dilution at 1:10 with PBS (0.14 M NaCl, 0.003 M KCl, and 0.01 M KH_2PO_4 -- Na_2HPO_4, pH 7.2) permitted kinetic attachment of single virions and aggregates to aluminized films for electron microscopy (4). Four pictures were taken of such a dilution and were divided into 5 equal areas, and counts of single particles, pairs, triplets, etc., were made on all 20 areas. A part of one picture is shown in Fig.25a, and the group frequency data are shown in Table 1, where it can be seen that the mean singles count per area was 124 with standard error $\sigma = 15$, and a mean frequency of pairs was observed to be 3.4%. Inasmuch as 124 particles would exclude 0.7% of the total area of the picture, we estimated an average of 5 pairs per picture would have been produced by coincidence

(30). The expected accidental frequency of triplets and groups of four were not calculated since the number of pairs observed indicated that aggregation was quite low in this preparation. That this stock of virus was quite stable is indicated by the increase of only 2% in pairs over a period of 60 days.

Dilution of Poliovirus into water. When a poliovirus stock of 7 x 10^{11} particles per ml was diluted 10-fold into PBS or 0.14 M NaCl, it was revealed to be dispersed, as shown in Fig. 25a, but when the same preparation was diluted 10-fold into distilled water, the particles formed aggregates as shown in Fig 25b. These aggregates were made of up to several hundred particles, but most often consisted of 10-50 particles. The aggregates appeared to be tightly bound together and did not spread out flat, but rather remained piled up on the aluminized film.



Figs. 25a and b A poliovirus stock concentrate at 7 x 10^{11} virions/ml was diluted 10-fold in (a) PBS; (b) distilled water

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These aggregates were produced under the conditions of reduced ionic strength as revealed by the fact that there was a rather sharp cut-off level in ionic strength above which aggregation did not occur, and below which it did. This point was approximately 10 mM for phosphate buffer (ionic strength $\Gamma/2 = 0.02$), and 60 mM for saline ($\Gamma/2$ - 0.06). Thus the aggregation could occur with appreciable salts in the water. An increase in ionic strength, as by further dilution in PBS or 0.14 M saline, led to the dispersion of these aggregates, and EM examination of these preparations revealed a picture similar to Fig. 25a.

⑰ 18X

⑰ ⑱ Blank

The formation of these aggregates was markedly dependent on particle concentration. When a preparation of poliovirus was diluted 1:10 into distilled water, aggregates formed as described above. However, when the same preparation was diluted 1:100 or 1:1000 into distilled water, the preparation remained dispersed, as revealed by a single particle approximation test as described above.

AGGREGATION OF POLIOVIRUS BY IONIC INVOLVEMENT

Three cations, Na^{+}, Mg^{++}, and Ca^{++}, were chosen for examination for their effect on poliovirus aggregation because of their physiological importance and wide-spread presence in water.

Effect of NaCl. An increase in sodium chloride concentration of up to 5.0 M had little effect on poliovirus, and led only to the formation of small numbers of pairs, triplets and aggregates of 4, 5, and 6 virions.

Effect of $MgCl_2$. Dilution of poliovirus to 7×10^{10} particles/ml in $MgCl_2$ of up to 0.25 M had effects similar to those of Na^{+}, and resulted in very little aggregation.

Effect of $CaCl_2$. Dilution of poliovirus to 7×10^{10} particles/ml in $CaCl_2$ of 0.001 M did not produce aggregation, but at 0.01 M aggregation occurred as shown in Fig 25c. (Higher concentrations of $CaCl_2$ could not be tested due to the formation of insoluble $Ca_3(PO_4)_2$ with the residual phosphate in the virus sample).

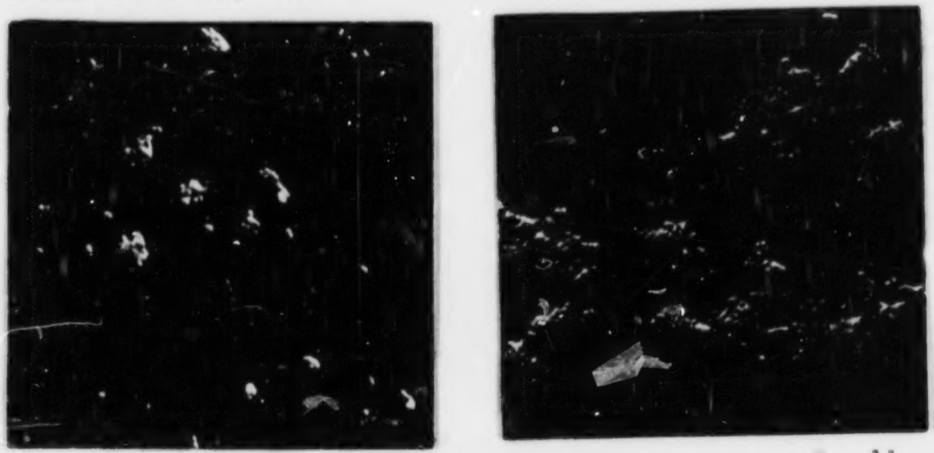
Fig. 25c Poliovirus stock concentration at 7×10^{11} virions/ml was diluted 10-fold in (c) 0.01 M $CaCl_2$.

3

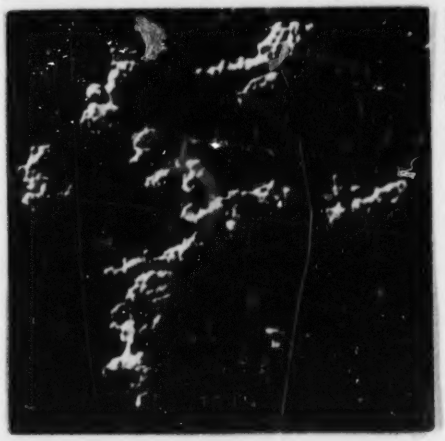
AGGREGATION OF POLIOVIRUS AT LOW pH

Quite important in the disinfection process is the effect of pH, particularly low pH, since drinking water processing involves lowering the pH with alum, and the subsequent raising with lime. Therefore, we investigated the effects of pH on aggregation in some detail.

Figures 25d, e and f show electron micrographs of aggregation of poliovirus as induced, respectively, at pH 6, pH 5, and pH 3. The aggregation at pH 6 is marked, but the aggregation at pH 5 and 3 was so massive that very few single particles could be seen in pictures taken at these pH values. This aggregation was quite similar to that shown for adenovirus-associated virus under low pH conditions by Johnson and Bodily (31).



Figs. 25d,e and f A poliovirus stock concentration at 7×10^{11} virions/ml was diluted 10-fold in (d) 0.05 M phosphate, pH 6.0; (e) 0.05 M acetate, pH 5.0; and (f) 0.05 M glycine-HCl, pH. 3.0. All were prepared for EM examination by the kinetic attachment method. Magnifications (a) 24,800 X, (b) - (f) 29,000 X.



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The SPA test was used in the investigation of aggregation by low pH since it allowed a kinetic curve to be established for examination of the rate of aggregation of the virus, as well as providing a measure of the total amount of aggregation that took place. The tests were conducted as follows. A stock solution of virus was diluted to various particle concentrations in a final volume of 0.5 ml in the buffer at the pH under study, in a 1/2 x 2 inch (1.27 x 5.08 cm) cellulose nitrate centrifuge tube. The tube was allowed to remain at room temperature (24C) for 60 minutes. Similar dilutions in other tubes were made and also incubated at room temperature for 40, 20, 10, and 5 minutes. The aggregation in each tube was halted by the addition of 4.5 ml of the same buffer at 20C, and the contents of each tube was thoroughly mixed. A sixth tube at pH 7 in 0.05 M phosphate buffer was included in each test to serve as a control titer of a non-aggregated preparation, since poliovirus did not aggregate in this buffer. All 6 tubes were centrifuged in a DuPont-Sorvall OTD-2 ultracentrifuge equipped with the "Reograd" control for termination of the run. It was found to be extremely important to allow the rotor to come to a slow, coasting stop, to prevent mixing within the tubes from destroying the separation of aggregate sizes established during centrifugation. This is critical because there is no density gradient present. The top 2.3 ml of each tube was then removed as carefully as possible with a 5 ml pipette, thoroughly mixed, and titrated on HEp-2 cells. The results of each titration were plotted as the logarithm (base 10) of the ratio of the titer in the low pH tubes to the pH 7 control.

Figures 26, 27 and 28 show kinetic curves of poliovirus aggregation at pH 6, 5 and 3 respectively. Each curve could be divided into two phases. The first phase was an initial rapid aggregation as the virus was first subject to the low pH conditions; the second phase, usually after 20 minutes, was a more level, stable phase wherein very little further aggregation took place. Under the conditions where aggregation took place, the appearance of large aggregates must have been the result of the clumping of single particles. As the aggregates formed, the remaining single particles were left further apart, making the formation of aggregates less likely. This is revealed by the fact that curves produced by more dilute preparations of virus show less rapid aggregation during the first phase, and a level second phase at a higher ratio value.

Additionally, these curves show that the amount of aggregation, revealed by the residual single particles in the top half of the centrifuge tube, becomes greater with decreasing pH. This is consistent with electron microscope data at pH 6, 5, and 3 which have revealed much more massive aggregation, and fewer single particles at the lower pH values (pH 5 and 3) than at the higher one (pH 6).

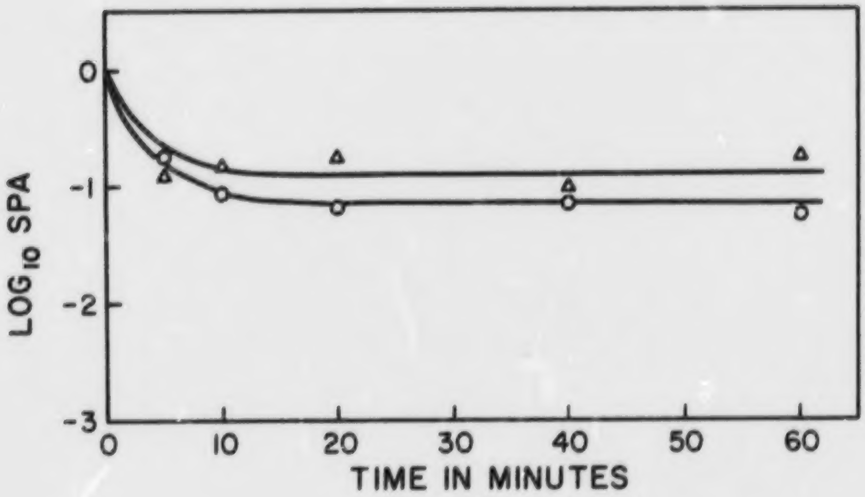

Fig. 26 Kinetics of aggregation of poliovirus at pH 6.0 in 0.05 M phosphate buffer as determined by the SPA test. Initial single particle concentrations: triangles, 1.4×10^{10}; circles, 7×10^{10}.

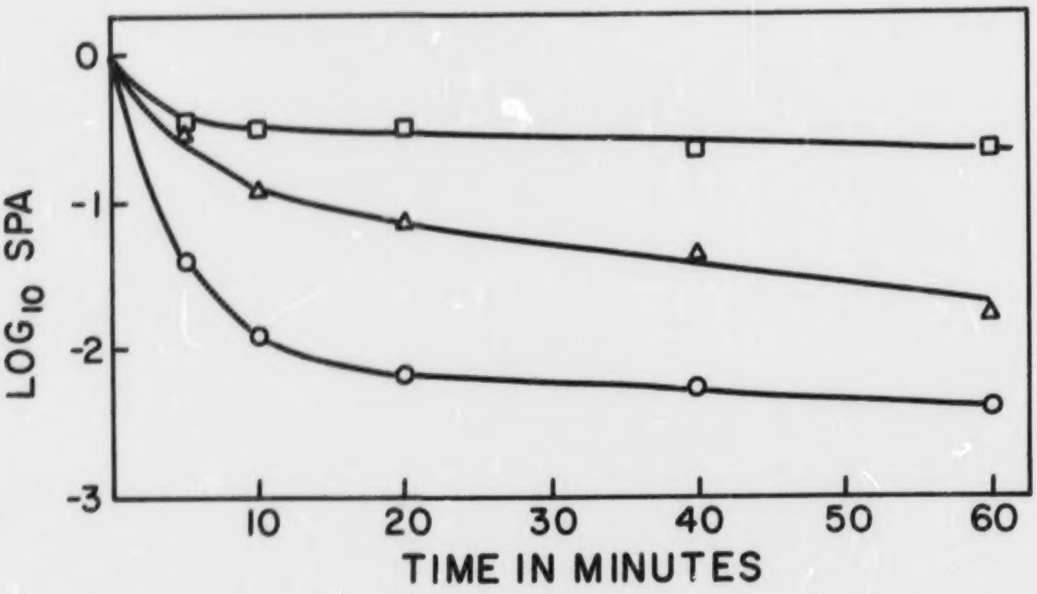

Fig. 27 Kinetics of aggregation of poliovirus at pH 5.0 in 0.05 M acetate buffer. Initial single particle concentrations: squares, 1.5 x 10^{10}; triangles, 3 x 10^{10}; circles, 1.5 x 10^{11}.

All aggregation experiments at low pH were performed in buffer, each at a concentration of 0.05 M. This aggregation was found, however, to be sensitive to the ionic strength of the solution, and could be reduced or prevented by addition of appropriate concentrations of NaCl or $MgCl_2$. Table 2 shows the inhibition of poliovirus aggregation at pH 6, 5, and 3. As the H^+ ion concentration was increased, higher concentrations of monovalent Na^+ ion were required to prevent aggregation, for example 2.5 M at pH 3 compared to 0.1 M at pH 6. The concentrations of divalent cation Mg^{++} required to prevent aggregation were generally lower than that of Na^+ at any given pH, (except for pH 5) but reached a plateau of 0.25 M at pH 5 and pH 3. Higher concentrations produced no further change in the state of aggregation.

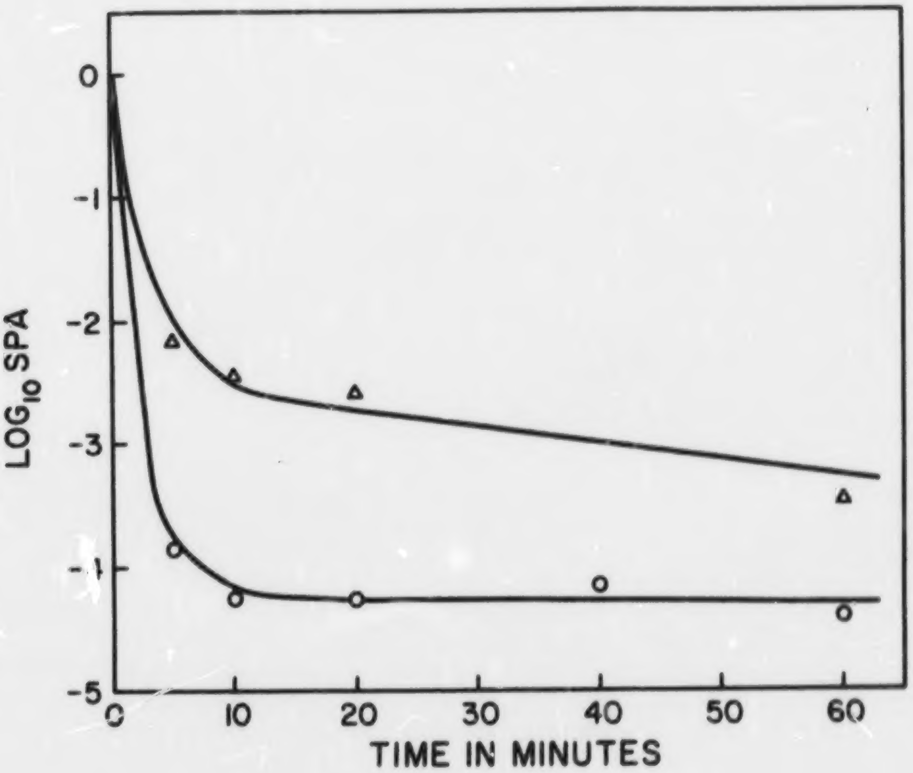

Fig. 28 Kinetics of aggregation of poliovirus at pH 3.0 in 0.05 M glycine-HCl buffer. Initial single particle concentrations: triangles, 7×10^9; circles, 7×10^{10}.

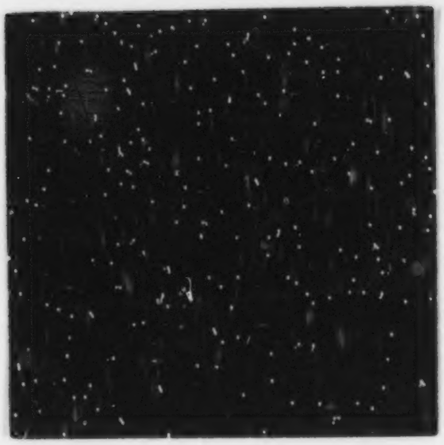

Fig. 29a Reovirus stock at 2 x 10^{10} virions/ml diluted (a) 200-fold in PBS.

The alkaline range of pH had little effect on the aggregation of poliovirus. At pH values up to 11, no significant aggregation took place, although poliovirus viability was found to be sensitive to pH 11, and lost plaque titer at approximately 1 $\log_{10}$ per hour. This is in contrast to low pH where poliovirus viability was found to be stable at pH 3 for periods of time up to 1 hour.

Aggregation at low pH was found to be a reversible phenomenon. Thus, when aggregates at pH 5 were returned to pH 7 by the addition of an equal quantity of pH 9 borate buffer, they broke up into single particles and small aggregates, and presented a picture similar to Fig 25a.

AGGREGATION OF REOVIRUS

The physical state regularly seen with reovirus concentrates prepared by Freon extraction and sedimentation velocity banding in sucrose is shown in Fig 29a. This picture was obtained when the stock virus in 30% sucrose was diluted into PBS in two steps: 10-fold, then immediately 20-fold; it was then treated for EM count by sedimentation on agar for pseudoreplication. The number of single particles, pairs, etc., are plotted as circles on the frequency chart, Fig 30. The single particles comprised about 79% of a total of 1.5 x 10^{10} virions per ml in this preparation, and was a consistent percentage in each preparation. This type of preparation is similar to that shown as the dotted line in Fig. 16 in which a crude, unpurified preparation of virus was subject to sedimentation velocity centrifugation in a zonal rotor (B-XIV). Both the purified and crude preparations had few aggregates of the smallest sizes, i.e. pairs, triplets, and so forth.

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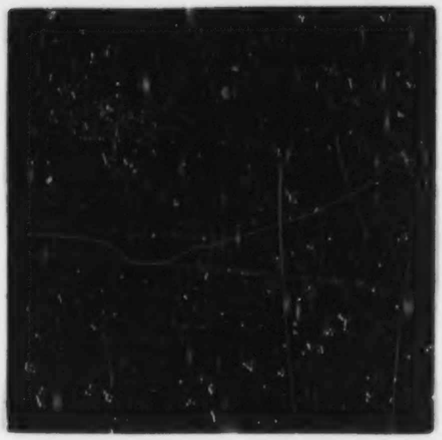


Fig. 29b Reovirus stock at 2 x 10^{10} virions/ml diluted (b) 10-fold in distilled water, allowed to remain at room temperature for EM examination by the spin-down method.

A markedly different picture was obtained when the stock solution was diluted first 10-fold into water or PBS and allowed to stand at room temperature for 2-3 hours, then further diluted 20-fold into PBS. These conditions produced the aggregation seen in Fig. 29b, and it is apparent that, in contrast to poliovirus, the aggregates did not disperse when diluted further with PBS in the final 20-fold dilution. Their frequency is plotted as squares in Fig 30. The percentage of single particles has dropped to 29% of the total. Freshly prepared stock concentrates of reovirus containing 10^{10} or more particles per ml regularly aggregated in water or PBS as described above, but they gradually lost this property, and after about 2 weeks of storage at 4-6°C they failed to aggregate under the above conditions.

The physical state of the virus in the undiluted concentrate cannot be examined by EM, but pictures of virus deposited on an aluminized collodion film from a 5-fold dilution in PBS were similar to Fig. 29a, and the frequency distribution plot was the same as the circles in Fig 30. Thus, a 5-fold or 200-fold dilution made no significant difference in the state of aggregation.

AGGREGATION OF REOVIRUS BY IONIC INVOLVEMENT

Reovirus behaved in a manner similar to poliovirus with respect to aggregation in NaCl, $MgCl_2$ and $CaCl_2$.

Effect of NaCl: No significant aggregation occurred in NaCl solutions up to 1.0 M.

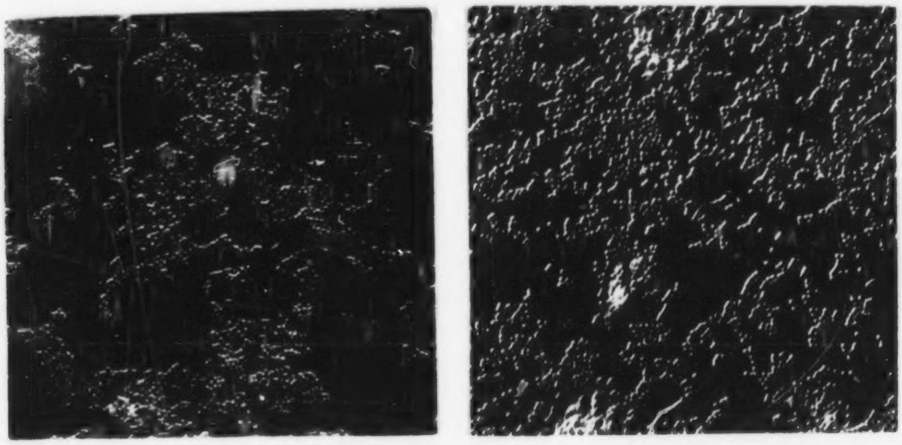
Effect of $MgCl_2$: Solutions up to 0.25 M have not produced any significant aggregation over the control preparation.

Effect of $CaCl_2$: Reovirus (at 2 x 10^{10} particles/ml) aggregated into groups of up to 100 particles at a $CaCl_2$ concentration of 0.01 M, but no significant aggregation occurred in $CaCl_2$ at 0.001 M.

6

AGGREGATION OF REOVIRUS pH: (SPA Tests)

Aggregation due to lowered pH took place with reovirus in a manner similar to poliovirus, with the exception that no significant aggregation occurred at pH 6. Figs 29c and d show electron micrographs of aggregates of reovirus at pH 5 and pH 3 as prepared by the KA method. As with poliovirus, the aggregation was quite marked at these low pH values. SPA tests were performed with reovirus as described above to measure the kinetics of aggregation at low pH. Figs 31 and 32 show kinetics of aggregation of reovirus at pH 5 and 3. The curves are similar to those of poliovirus, and likewise can be divided into two phases, the first, a rapid decrease in single particle titer and the second, a leveling off after 10-20 minutes with little or not further increase in aggregation. At pH 5, the kinetic curves (Fig. 31) show that at a higher particle count, aggregation was more rapid than at lower concentrations, consistent with the results found for poliovirus. However, at pH 3 (Fig 32) the aggregation kinetic curves show that the most concentrated virus preparation (4.6×10^{10} particles/ml) gave the least reduction in titer due to loss of single particles into groups, while the least concentrated preparation (1.8×10^{9}) gave the greatest aggregation. The reasons for this apparent reversal of behavior are unknown, but are at present under investigation.



Figs. 29c and d Reovirus stock at 2×10^{10} virions/ml diluted (c) Stock at 4.6×10^{11} virions/ml diluted in 0.05 M acetate at pH 5.0 (d) Same stock as (c) diluted in 0.05 M glycine-HCl, pH 3.0. Particles in (c) and (d) were collected by the kinetic attachment method. Magnifications: 8,500X.

⑲ 20X

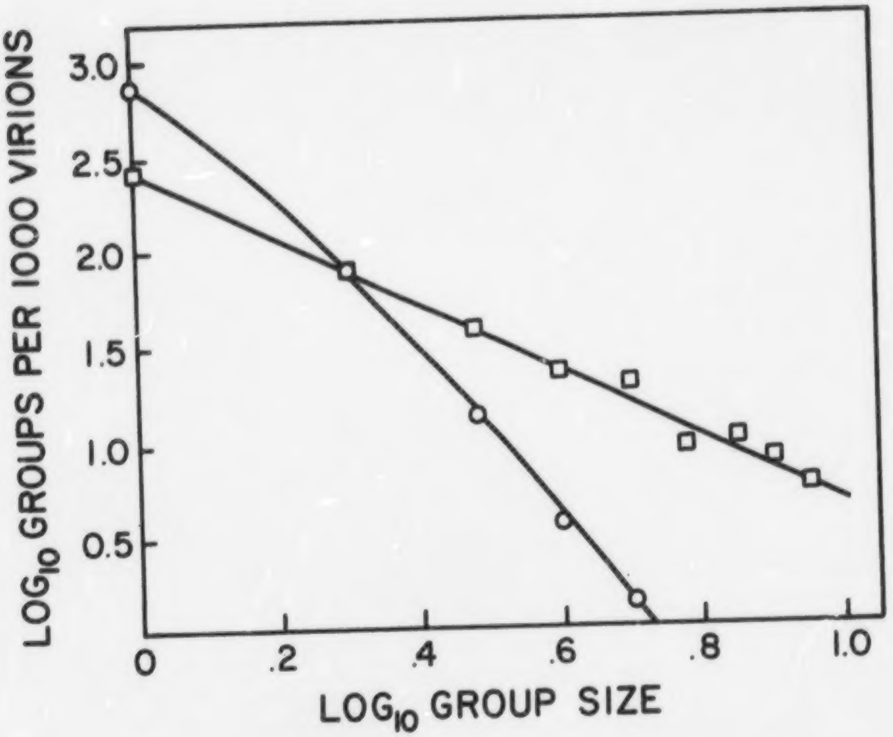


Fig. 30 Electron microscopic evidence of reovirus aggregation on dilution with distilled water.

As with poliovirus, aggregation of reovirus at low pH was sensitive to the ionic environment. Table 2 shows the minimal amount of NaCl and $MgCl_2$ required to prevent aggregation at pH 5 and pH 3. Somewhat larger concentrations of Na^+ are required to prevent aggregation at pH 5 for reovirus than for poliovirus, 0.6 M as compared to 0.2 M, but Mg^{++} inhibited aggregation at pH 5 at 0.25 M for both viruses. Aggregation at pH 3 required larger concentrations of Na^+ than at pH 5 for inhibition, but the concentration of Mg^{++} required was the same as for pH 5 inhibition: 0.25 M. Higher concentrations of Mg^{++} ion at pH 3 produced massive aggregation; these were the only conditions under which Mg^{++} ions caused aggregation of reovirus.

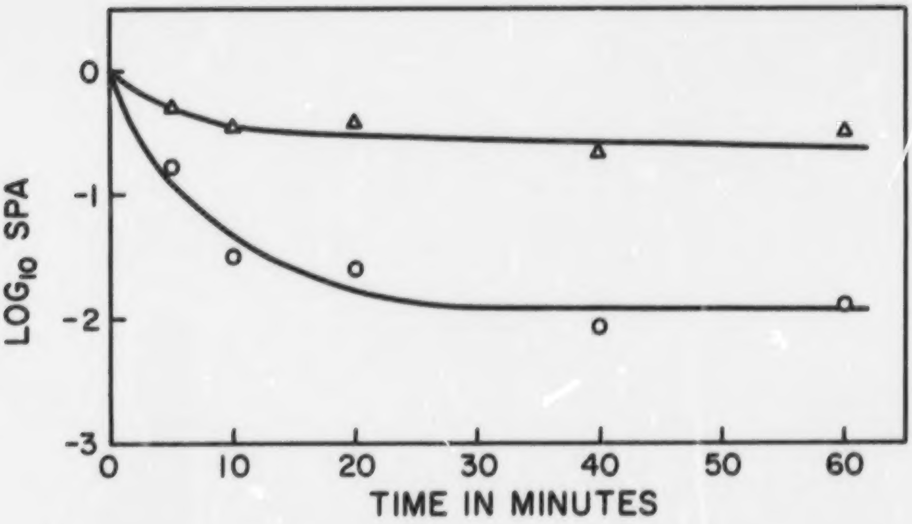


Fig. 31 Kinetics of aggregation of reovirus at pH 5.0. Initial single particle concentrations: triangles, 4.6×10^9; circles, 1×10^{10}.

Aggregation at pH 9, 10 and 11 was minimal with reovirus and was not significantly greater than that of the control of pH 7. Viability was sensitive to pH 10 and 11, and the plaque titers fell at the rates of 0.52 $\log_{10}$/hour and 0.58 $\log_{10}$/hour, respectively.

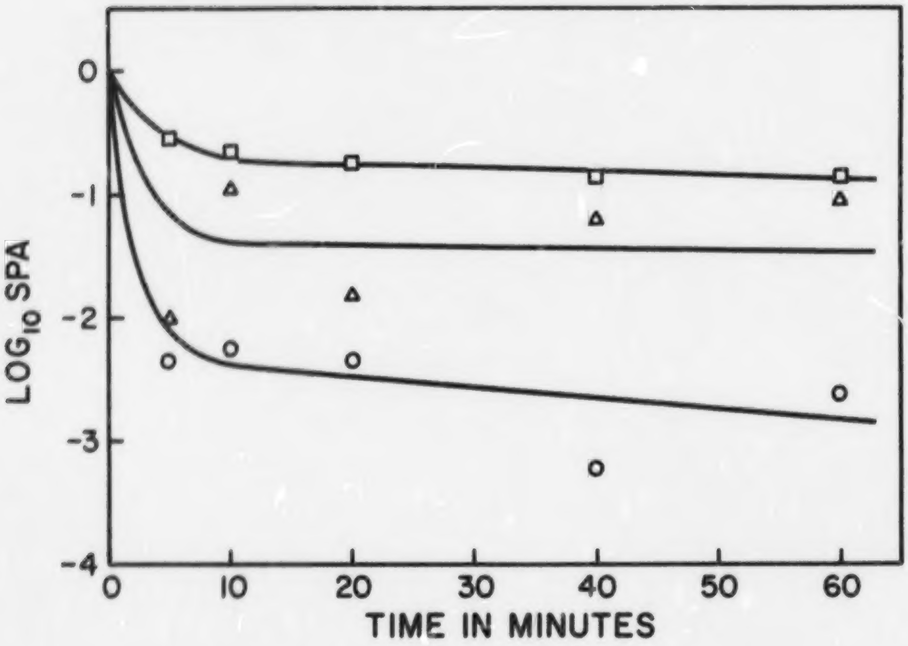


Fig. 32 Kinetics of aggregation of reovirus at pH 3.0. Initial single particle concentrations: squares, 4.6×10^{10}; triangles, 1.8×10^{10}; circles, 1.8×10^{9}.

POLIOVIRUS AGGREGATES AND THEIR SURVIVAL IN WATER (24)

Rate Zonal Analysis of sedimenting Plaque Forming Units (PFU) in Density Gradients of Sucrose Dissolved in Water. Infected cells were harvested in distilled demand-free water and the polio virus released by two freeze-thaw cycles. This crude lysate titrated 8.7×10^{8} PFU/ml and 15 ml of a 2-fold dilution of it were layered over a 15-30% w/w sucrose gradient in an Oak Ridge type BXIV Zonal rotor (23) and centrifuged at 32,000 RPM and 20C for 112 minutes ($w^2t = 7.95 \times 10^{10}$). The sucrose solutions were made with distilled water containing no buffers or other salts. Fractions were cut from the gradient and

no buffers or other salts. Fractions were cut from the gradient and

the plaque titer of each is plotted (open circles) on Fig. 33 where it can be seen that there is no sharp peak anywhere. A broad band of infectivity extends over fractions 14 to 21 but there is no sharp maximum in the region of fraction 9 where single virions would be expected in this run. The total of all PFU recovered from all the fractions was 20% of that put in. Apparently 80% of the PFU have sedimented to the rotor rim. This experiment was repeated several months later with virus prepared the same way with the same results.

Another zonal centrifuge run was made with well-dispersed purified polio virus. This stock virus had been stored in 0.05 M phosphate buffer at pH 7.2 containing 20% sucrose. It was diluted with an equal volume of PBS and 15 ml was layered over the 15-30% w/w sucrose gradient and centrifuged exactly as above. The total particle count in the 15 ml starting volume was 3×10^{11} which yields approximately the same PFU input as that employed in the crude virus experiment above. Plaque titers of these fractions are plotted (filled circles) also on Fig. 33 and now a small sharp peak appears where none was before, in fraction 9. There is no peak in the deeper region where most PFU appeared in the crude preparation but substantial numbers of PFU were found at all levels in the density gradient indicating that aggregation was present to an extent much greater than that present in the stock virus (Fig 19).

A third zonal centrifuge run was made, this time with purified virus in a 15-30% w/w sucrose gradient containing PBS (those above contained no salt). The PFU were found in a very narrow band. One fraction contained 63% with 35% divided about equally between the 2 on either side. The combined titer of all the other fractions was only 2%. This virus was certainly well dispersed.

AGGREGATED VIRUS AND ITS SURVIVAL IN THE PRESENCE OF BROMINE

One way to get all the virus (singles and aggregates) from a crude cell lysate and still render it sufficiently free of halogen demand for disinfection experiments is to pellet it repeatedly in the centrifuge discarding soluble material in the supernatant fluids. Unfortunately this process tends to compound the ambiguity of interpretation by inducing aggregation among even those virions that might have been free and promoting added complexity with particles of foreign matter. We have layered crude cell lysate (freeze-thaw) over a 5% sucrose solution in a swinging bucket centrifuge tube (SW-27 rotor) and sedimented the virus into a pellet (25,000 RPM, 4 3/4 hours at 4C). This should leave all the soluble material near the top of the supernatant fluid and it did. Fifty fold dilutions of the resuspended pellet material had no appreciable bromine demand. Still there was enough particulate

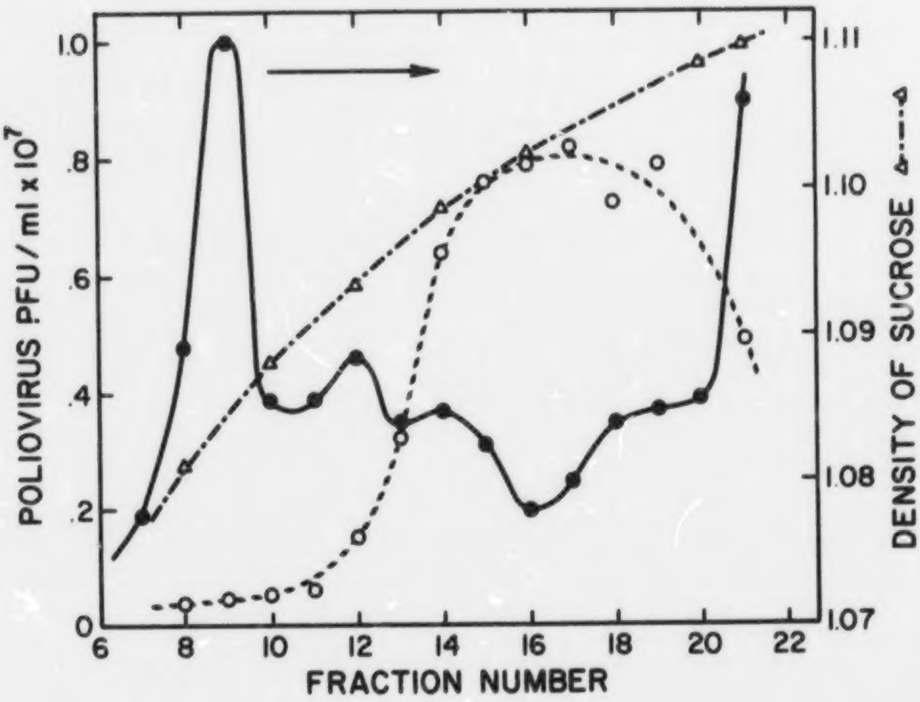


Fig. 33 Zonal centrifuge velocity analysis showing poliovirus PFU due to single virions and aggregates.

foreign matter present to make any quantitative estimate of virion aggregation by electron microscopy impossible. Exposure of this virus to 10 μM HOBr at pH 7 and 10C revealed the strong influence of aggregation (Fig. 34, triangles). The initial slope of the semi-log plot of the survival ratio is approximately the same as that for single particles showing that many single virions must have been present but after exposure of 12 seconds to the bromine there were 110 times more survivors in the pelleted virus than there were in the dispersed preparation (circles, Fig. 34).

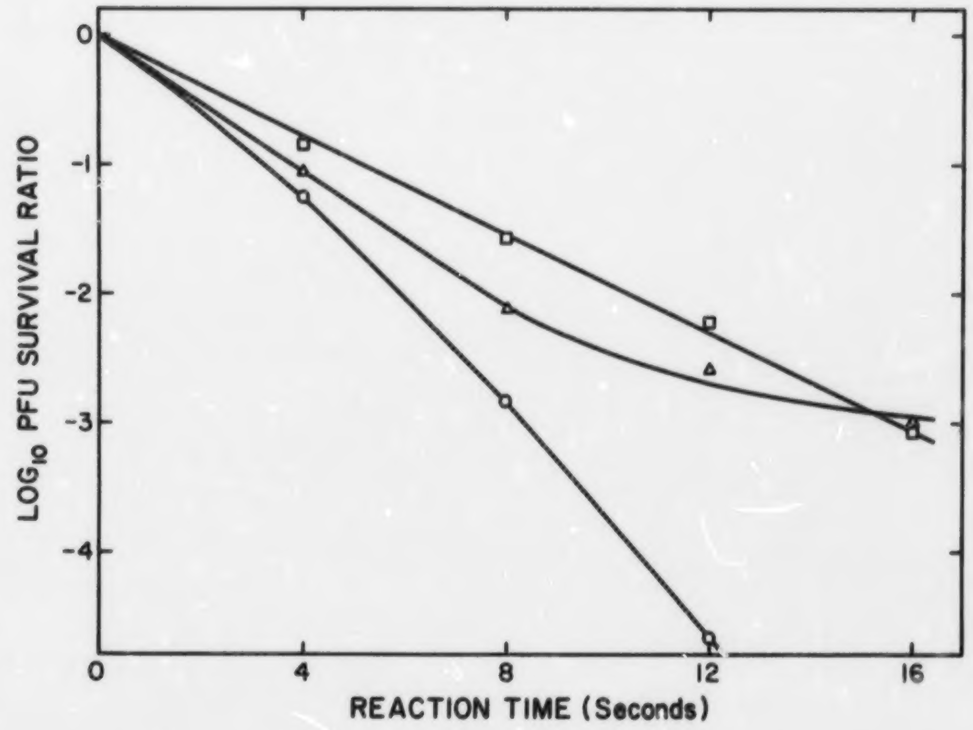


Fig. 34 An adventitious mixture of singles and aggregates can give a straight line disinfection plot.

In order to avoid whatever virion aggregation might be induced by pelleting and still minimize halogen demand we dialyzed about 100 ml of freeze-thaw cell lysate in the Amicon Model 202 Ultrafiltration Cell using an Amicon XM 300 filter disc and continuous agitation while passing through 2 liters of demand-free water. With a nitrogen pressure of 30 psi this took 24 hours at 4-6C. On exposure to 10 μM bromine the $\log_{10}$ PFU titer of survivors was quite linear while it declined by a factor of 1000 (Fig 34, squares). After 12 seconds of exposure the survivor titer in the dialyzed virus preparation was 288 times greater than that found in similarly treated dispersed virus. This clearly demonstrates that a linear semi-log decline in survival titer can be obtained with aggregated virus but the slope, of course, is very much less than that for singles. Independent evidence of aggregation in this preparation was provided by electron microscopy, Figs. 35, 36, and 37 showing a few single virions, aggregates associated with foreign material and multi-layer virion aggregates completely free of other material. We were not able to obtain a meaningful frequency distribution chart of aggregate sizes in this preparation but it must have been different from that in the pelleted virus.

RESPONSE OF POLIOVIRUS AGGREGATES TO ACOUSTIC ENERGY

Attempts to disperse aggregated polio virus with acoustic energy (sonic waves at 20,000 Hz) have not been very successful. The Branson Sonifier Model LS-75 manufactured by Heat Systems Inc., of Plainview, N. Y. with the "Micro Tip" was used and the rate of energy input to the sample was determined by measuring the rate of increase in temperature it produced in 5 ml of water in a well insulated test tube. This proved to be quite reproducible and we used the dial setting #1 that gave about 2.5 calories per second or 10.5 watts. This power level heats small samples very quickly and inactivates the virus before observable deaggregation takes place if the temperature rises much above 40°C. Heating the virus to 50°C for 30 seconds in a water bath produced a comparable loss in titer. However, the same 10.5 watt power input produced no loss in titer when the sample was kept in an ice bath so that the temperature did not rise above 31C during the treatment. In spite of this high level of acoustic power absorption in a 5 ml volume of virus suspension for 30 seconds, clumps of 100 or more virions could still be found by electron microscopy. In one case a 6 fold increase in titer was observed when bromine treated survivors were sonic treated at the 10^{-3} survivor level but in several other instances no changes occurred. Sonic treatment of aggregated poliovirus before treatment with bromine did not alter its inactivation rate.

THE SPECTRUM OF AGGREGATE SIZES IN A MIXTURE, SEPARATED INTO 3 ARBITARY PARTS.

The above experiments were made with virus preparations containing at least 10^{10} virions (ca 10^{8} PFU) per ml. This was done in order that sufficient virus would be present to permit direct observation by electron microscopy. The physical state of poliovirus at low concentrations in water is not observable directly by electron microscopy and PFU titration of the many fractions from rate-zonal centrifugation is a laborious process. Therefore we have devised an abbreviated rate-zonal experiment that gives an approximation of the percentage of single virions in the preparation as well as a measure of the frequency of small and large aggregates.

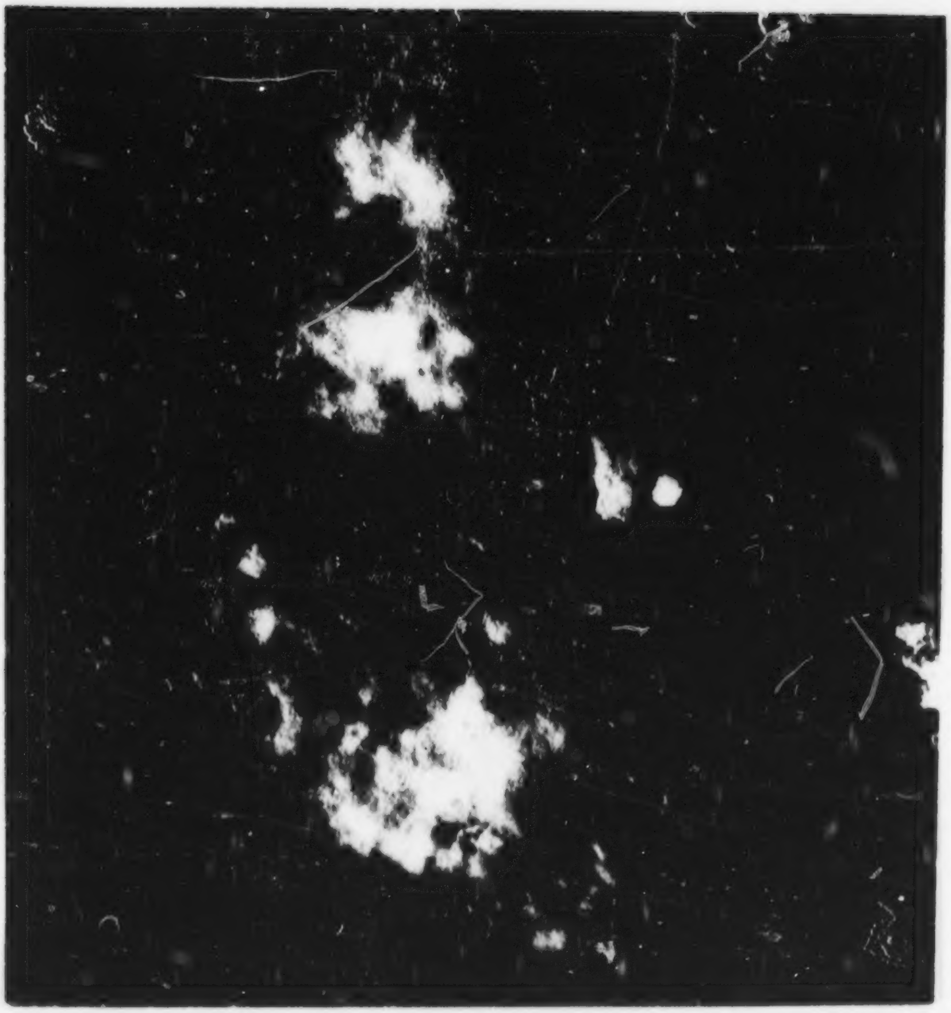


Fig. 35 Typical poliovirus aggregates found in a dialyzed but purified freeze-thaw lysate of infected HEp2 cells.

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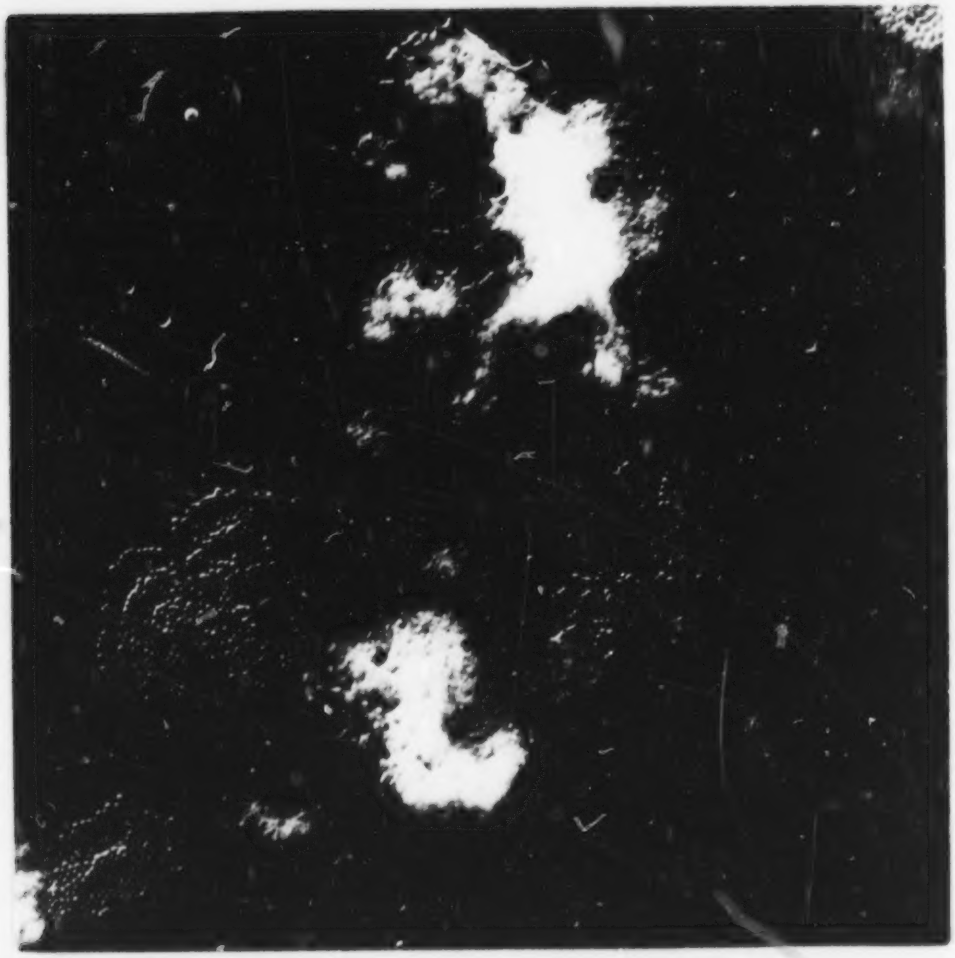

Fig. 36 A closer view of poliovirus aggregates mostly associated with foreign material probably host cell debris.

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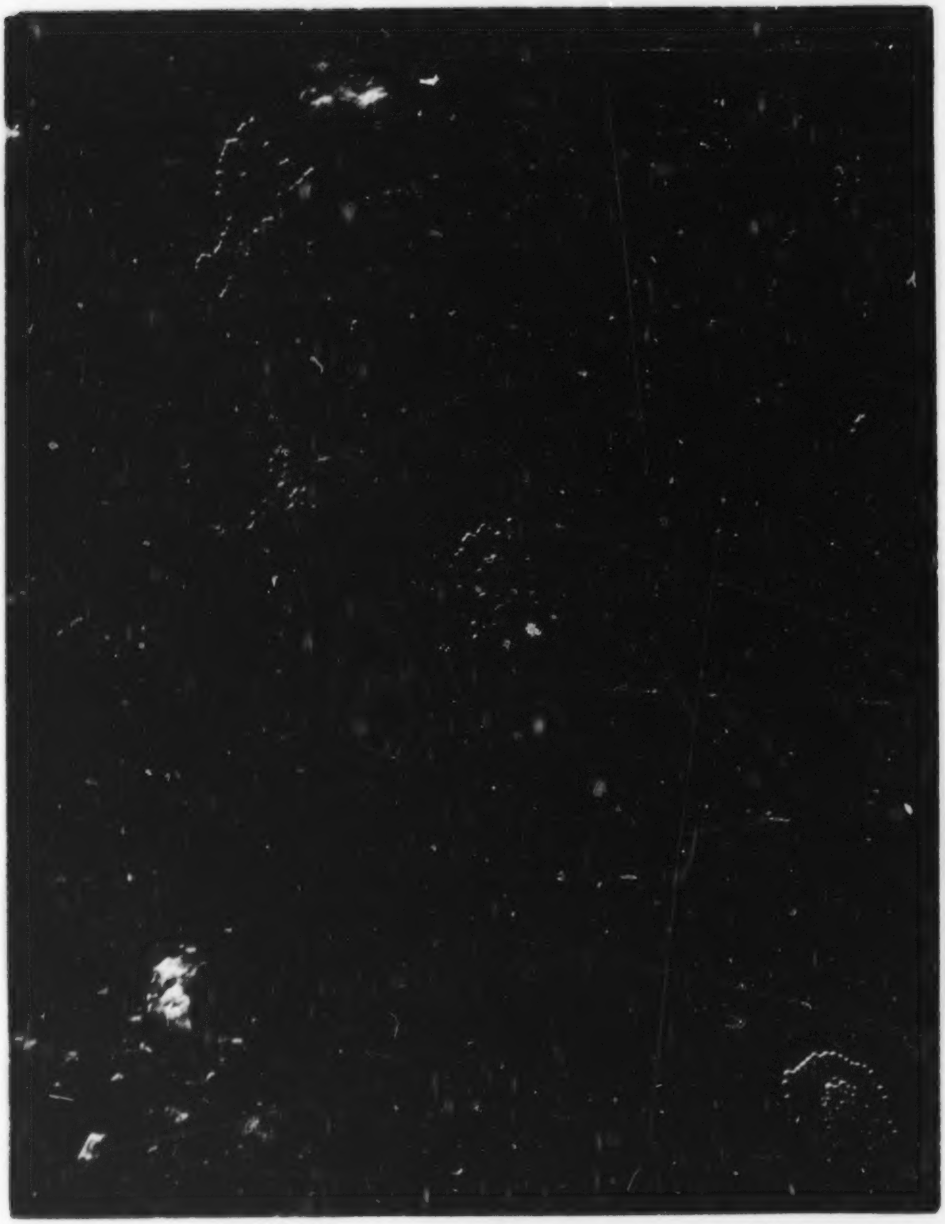

Fig. 37 Some poliovirus aggregates, even in unpurified lysates of infected cells, appear to have been reconstituted from dispersed single particles.

㉑ 22x

The bicenterial experiment (devised in this year of 1976) employes 3 different layers of fluid density 2 of which are distinguished by blue and red dyes and the top one in the centrifuge tube is colorless (white). The details are shown in Fig. 4 in the foregoing section on Materials and Methods. There is nothing basically new about the theory of its operation. Its practical value is its main asset. Aggregates of spherical particles must sediment more rapidly than single spheres. Pairs sediment about 40% faster so the thickness of the top (white) layer is made just so that all the pairs suspended in it will have passed down into the middle (blue) layer when the centrifuge is stopped. The blue layer is more dense than the top layer so small aggregates will be retarded in their passage on down into the very dense red layer. Here the largest aggregates will gather. Many of them will not reach the bottom of the centrifuge tube by the time the run is done. Some will but even they will not form a very compact pellet because their density differs so little from that of the sucrose. Thus the spectrum of aggregate sizes is separated into 3 arbitrary parts. The actual group sizes found in each of these layers have not been measured but doubtless they could be calculated if suitable assumptions were made regarding the geometry of the clumps. What is probably more important at this point is the fact that six test tubes containing five unknowns and one control can be spun in this way to give a quick estimate of their relative degree of aggregation.

Two points must be emphasized. The titers of the 3 sections (red, white and blue) do not simply give the numbers of singles, small aggregates and large aggregates directly. In reading the tables of data, each sample must be compared with the unaggregated control. And the second point is that care must be taken with this test as well as with the similar SPA test (Fig. 3) to be sure that no mixing occurs during starting and stopping of the swinging bucket rotor. Our centrifuge is a Dupont-Sorvall with hydraulic drive (Model OTD2). If another machine is used, it must have suitable control of both start and stop to prevent mixing.

THE FATE OF POLIOVIRUS AGGREGATES WHEN DILUTED IN A LARGE VOLUME OF WATER.

The virus from two ml of a freeze-thaw lysate of infected cells was separated into 3 parts as described above. The two top fractions were discarded. The bottom red section provided a stable stock of aggregated virus. Dispersed virus of comparable titer was prepared by diluting a purified concentrate with PBS. The experiment consised of adding one drop of either the dispersed or the aggregated virus to 200 ml of the water sample under test. With constant mixing this approximately 7000 fold dilution was kept at 20C and a sample was removed after one minute and another after 60 minutes. Each of these samples was centrifuged again and the red, white and blue parts titrated separately.

Water of several kinds was tested in this way for its effect on the virus. From the Chapel Hill water purification plant, the incoming reservoir water was tested before and after centrifugation sufficient to remove particles of poliovirus size. Finished water from the purification plant (dechlorinated by ultraviolet light) and secondary effluent from the sewage plant (chlarified in the same way as the reservior water and unclarified) were tested as well as several controls including distilled, deionized water and PBS.

The results are shown in Table 3. In all the tests the plaque titer of the starting virus mixtures in the 200 ml reaction vessel was in the region of 10^4 PFU per ml. The degree of aggregation of the virions in each test can be judged from the fraction (percent) of titer in the white, blue, and red parts of each centrifuge tube.

Aggregated virus remained aggregated in distilled, reservoir and finished water, leaving only 7-15% in the white supernatant region and 43-74% in the aggregated red region. Dispersed virus remained dispersed in all the samples except the finished water from the filter plant. In this finished water only 6% of the PFU remained in the white supernatant layer. About 50% were in the small aggregate blue region and the rest were in the red bottom. This result was confirmed by 3 more similar tests. Either this virus has become aggregated or it has formed complexes with insoluble particulate matter in the finished water and so increased in sedimentation rate.

A surprising result was observed with PBS with the effluent from the sewage plant. In both of these the aggregated virus became dispersed so that the PFU titer in centrifuged supernatants was indistinguishable from the controls.

Some small changes can be seen (Table 3) when one minute samples are compared with 60 minute samples but it seems that most of the action has occurred within the first minute after the 7000 fold dilution whether it be aggregation or deaggregation. The one result, with aggregated input virus and unclarified sewage plant effluent, may be a significant exception. Here the evidence for continuing dispersal of aggregates appears as a 2 x titer increase from 1 to 60 minutes in the white supernatant and a simultaneous decrease (2 fold) in the red.

Two more controls were added in an attempt to learn the reason for the apparent dramatic aggregation of dispersed virus by finished water from the filter plant. Inasmuch as lime and alum are used in treatment of this water before filtration, we tested a calcium chloride solution containing 6.2 mg/l Ca^{++} (the concentration of Ca^{++} found in the finished water by atomic adsorption spectroscopy) and another dosed with 20 mg/l $CA(OH)_2$ and 32 mg/l of $Al_2(SO_4)_3.18\ H_2O$ (the dosage used at the Chapel Hill water treatment plant), adjusted dropwise to pH 7 with 1M HCl, and centrifuged to remove aluminum hydroxide floc at a speed sufficient to pellet particles of poliovirus size. Both solutions were allowed to stand 6 hours at 25C before addition of the virus. These data are included in the summary Table 3 where it can be seen that calcium alone had no effect but when the alum was present about 80% of the virus sedimented to the bottom.

The virions in the drop of concentrate may have aggregated instantly when they fell into the 200 ml of alum-treated water or they may have reassembled after the 7000 fold dilution or their high sedimentation rate may not be due to aggregation but rather to formation of dense complexes with particles already present in the water. Instant aggregation would have to be critically dependent on the virion concentration in the drop so a drop was put into 200 ml of distilled water (about 7000 x dilution). Previous experiments have shown that this diluted virus is dispersed. When 5 ml of this were mixed with 15 ml of alum-treated water and analyzed 15 minutes later only 8% of the PFU were found in the combined white and blue fractions; 92% sedimented to the bottom red fraction. This indicates complex formation rather than virion aggregation.

PHYSICAL STATE OF POLIOVIRUS RELEASED FROM INFECTED CELLS DIRECTLY INTO NATURAL AND OTHER WATERS.

Experiments above have investigated aggregation induced by diluting dispersed virus preparations into essentially salt-free or fresh water, sea water and into weakly buffered waters at a series of pH values. They were also concerned with the fate of aggregated virus clumps when dispersed in several kinds of water. In order to get one step closer to field conditions we have put infected cells into a series of waters and released the virus from them by two freeze-thaw cycles. The resulting virus concentration was contrived to be low (ca 10^4 PFU per ml), too low for electron microscopy but high enough for rate-zonal sedimentation analysis (RWB). Table 4 shows that only in PBS and in sea water was there essentially no virion aggregation in these suspensions. Distilled water showed very little aggregation but Chapel Hill reservoir water (both clarified and raw) showed a large aggregated component indicating that halogen treatment of such water must be designed to inactivate resistant aggregates rather than just the sensitive single virions.

POLIOVIRUS INACTIVATION BY BROMINE SPECIES OTHER THAN HOBr AND BY HOCl.

Tribromamine (NBr_3). In figure 38 are shown the kinetics of inactivation of singly dispersed poliovirus by several concentrations of NBr_3 at 5C in 0.05 M phosphate buffer at pH 7. The dependence of reaction rate on concentration is shown in Fig. 39.

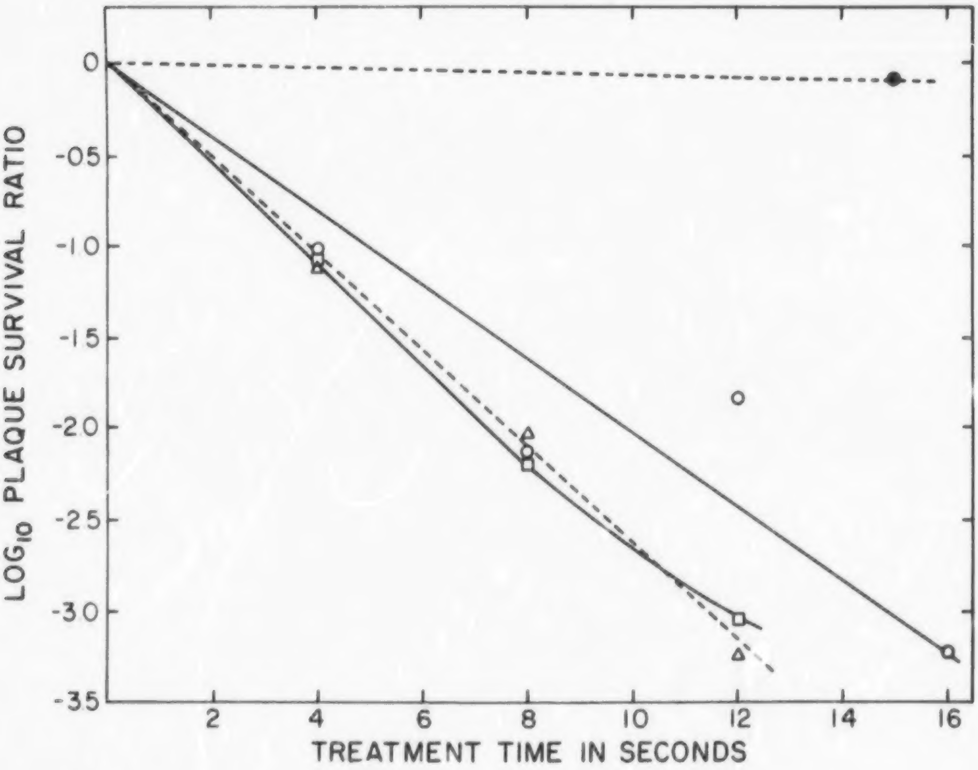

Fig. 38 Inactivation of poliovirus by tribromamine (NBr_3) at 5°C, pH7 and a concentration of 3.2 μM (top line), 12 μM (-Δ-Δ-Δ-Δ-), and 49 μM (—□—□—□—□—).

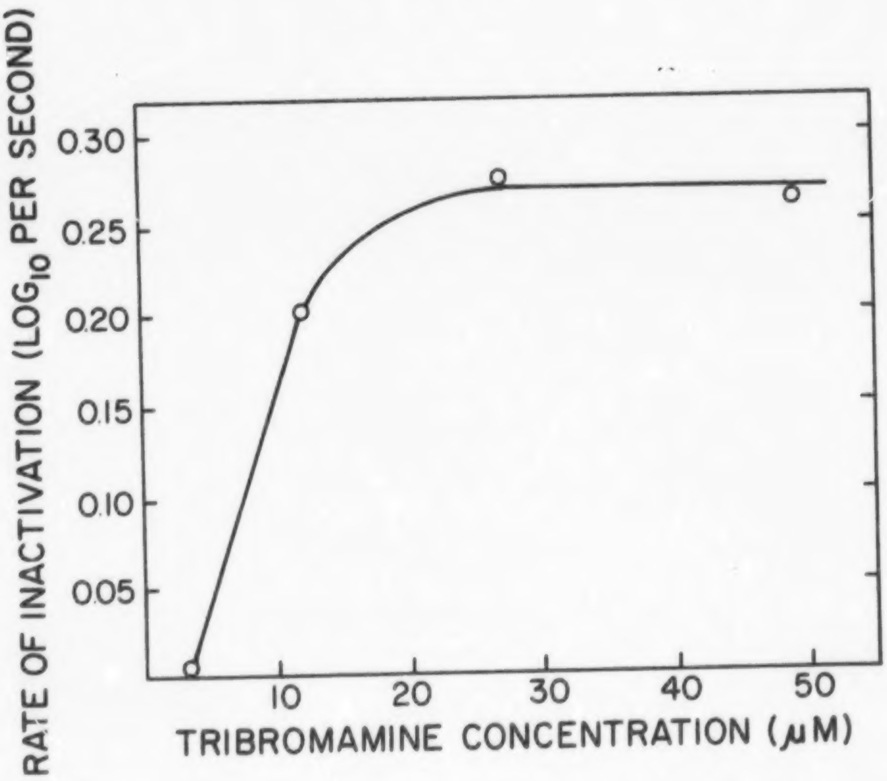

Fig. 39 Inactivation rate of poliovirus as a function of the concentration of NBr_3 at 5°C and pH7.

Dibromamine ($NHBr_2$). Poliovirus reacting at 4C with dibromamine at several concentrations is shown in Fig. (40). All experiments were at pH 7 in 0.05 M phosphate buffer in which we have not observed any tendency for dispersed virus preparations to aggregate. Still, the curved lines obtained in this particular set of tests would seem to indicate that aggregation was indeed present. We cannot account for this. Dibromamine is much slower in action than tribromamine at the same molar concentration. Note that the time scale is seconds for the former and minutes for the latter.

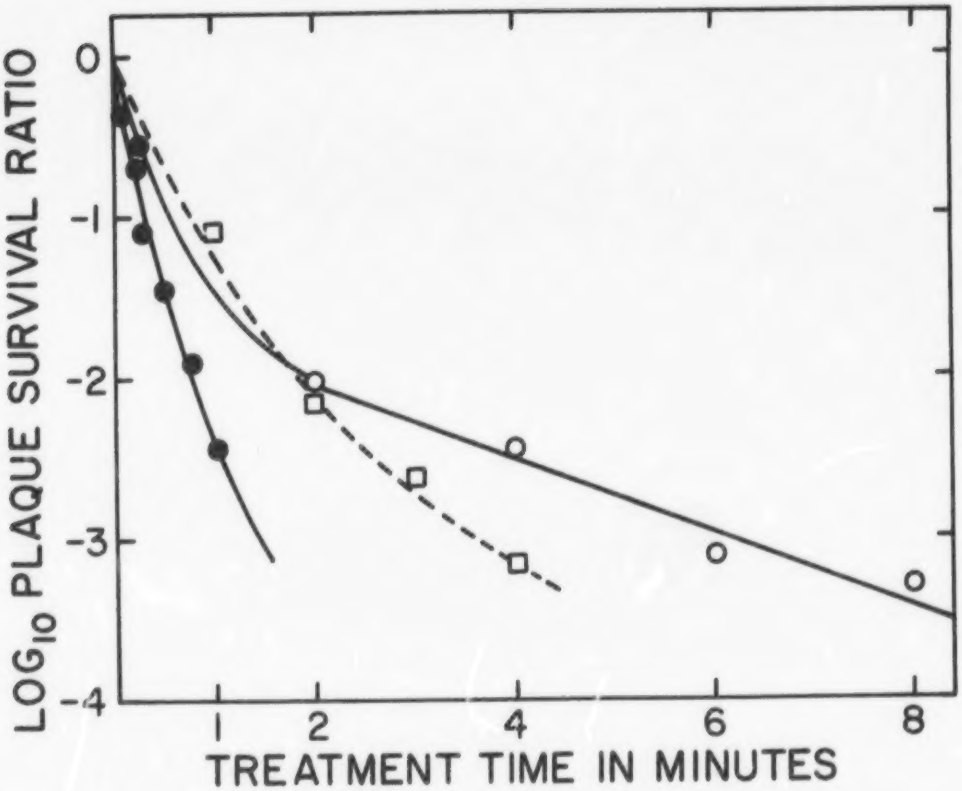

Fig. 40 Inactivation of poliovirus by dibromamine (NHBr$_2$) at 4^{o}C, pH7 and a concentration of 2.9 μM (-○-○-○), 13.8 μM (-□-□-□) and 39 μM (-●-●-●).

Br$_2$. Poliovirus is inactivated very rapidly at 4C by Br$_2$, much faster even than by HOBr (compare Figs. 41 and 23). In order that the Br$_2$ be the only significant species active in the experiment it was necessary to perform the reaction at pH 5. It would have been quite impossible to avoid aggregation of the virus at this pH had we not previously learned, as described above, that inclusion of 0.3M sodium chloride would prevent it. Poliovirus is entirely stable at pH 5 so it would not have declined in titer because of the acid conditions of the 0.05 M acetate buffer used. Inactivation rates at several concentrations of Br$_2$ are shown on the resume, Fig. 42.

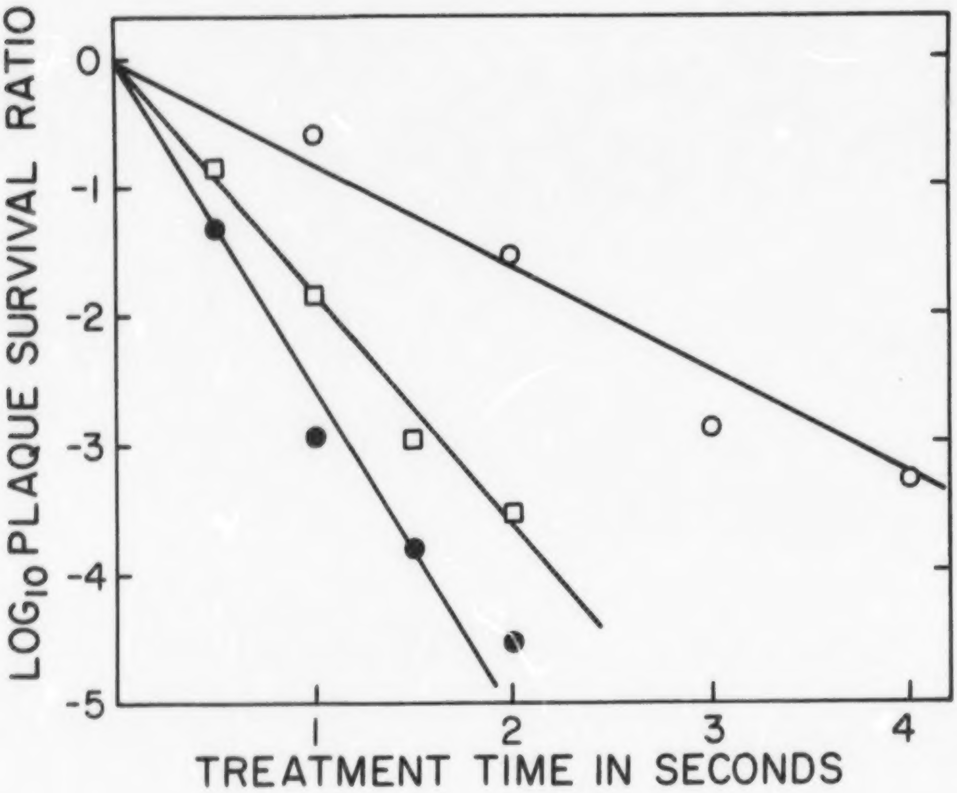

Fig. 41 Inactivation of poliovirus by Br_2 at 4°C and pH5 at 4.7 μM (-○-○-○-), 12.9 μM (-□-□-□-) and 21.6 μM (-●-●-●-).

OBr^-. The rate of inactivation of poliovirus by the OBr^- ion in 0.01 M glycine buffer at pH 10 and pH 11 is shown in Fig. 43. The initial rates at both pH values are roughly proportional to OBr^- concentrations but neither of them is constant and they are very much greater than those of HOBr at the same molar concentrations. Apparently the presence of a few aggregates that produced little curvature in the log plot of T/To for HOBr (28), tribromamine (Fig. 38) or Br_2 (Fig. 41) was more effective in progressively reducing the reaction rate with OBr^-. A final experiment was performed to examine this effect further. Starting virus was centrifuged to remove aggregates immediately before the disinfection

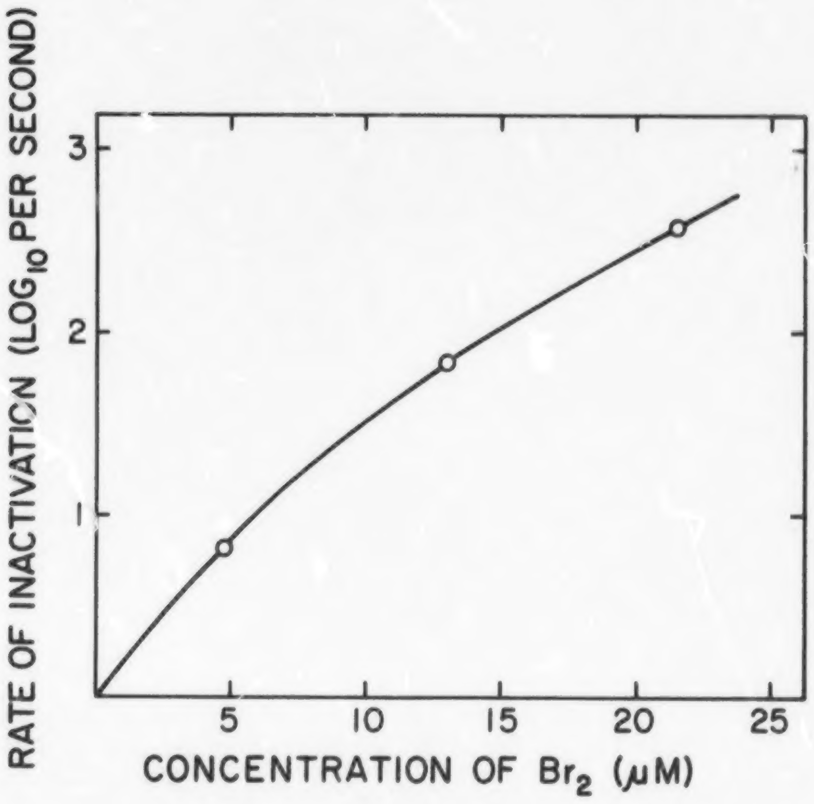

Fig. 42 Inactivation rate of poliovirus as a function of the concentration of Br_2.

experiment which was then repeated. The progress of the disinfection showed the same degree of curvature as before. It appears that this curvature is not due to virion aggregation and at present we have no other explanation for it. The rate of inactivation of poliovirus by OBr^- is about 10 times faster than that of an equal molar concentration of HOBr at 2C. No reduction in plaque titer has been observed with poliovirus at pH 11 during the short time required for these inactivation experiments.

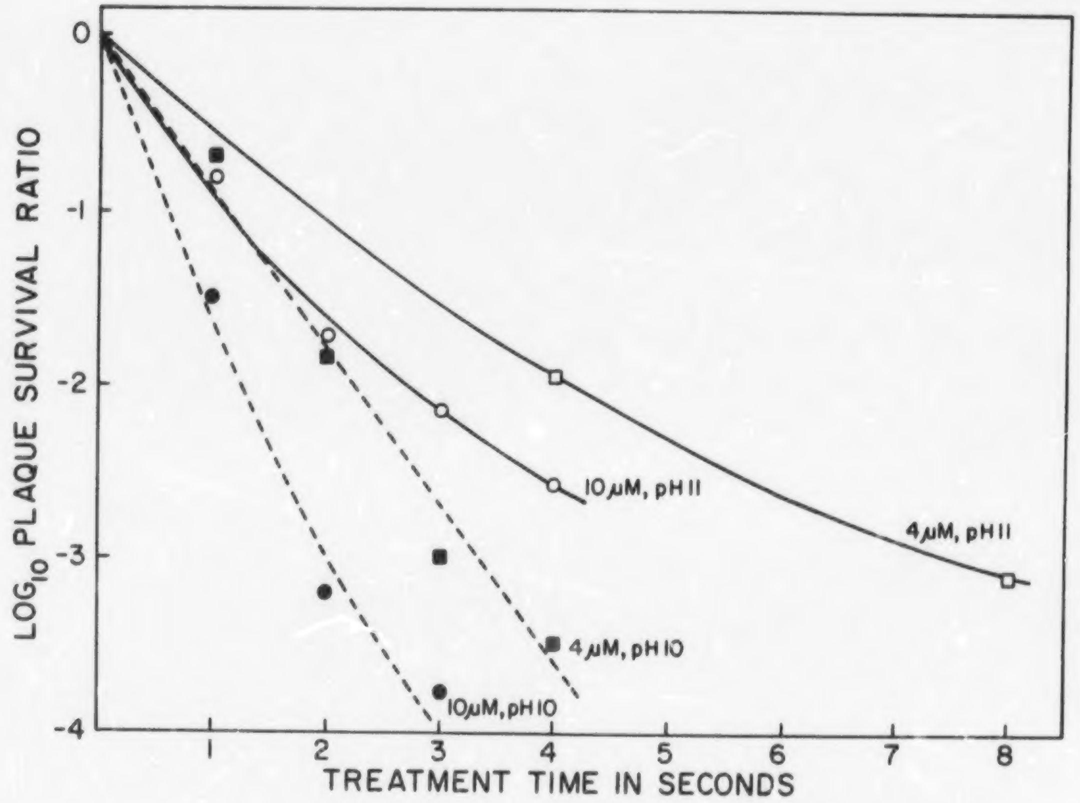

Fig. 43 Inactivation of poliovirus by OBr^-. The rates are approximately proportional to concentration at the two concentrations tested at both pH values but they are significantly greater at pH 10 than at pH 11.

HOCl. Inasmuch as data on the rate of inactivation of polio and other viruses has been obtained in the past without direct observational control of virion aggregation, we felt obliged to make some experiments that would provide directly comparable data. But at the time the chlorine experiments were made we were not aware of the fact that dilution of stock virus with dilute buffer at pH 6 caused virion aggregation. Experiments with HOBr were made at pH 7 where there was no aggregation but with HOCl we worked at pH 6 and the results were highly irregular, some experiments gave straight lines (log survival titer vs time), others curves. Although these results indicate that chlorine inactivates poliovirus more slowly than equimolar concentrations of bromine, these data are not yet complete enough for publication. Now that we have learned how to prevent aggregation (29) of the virus at acid pH these chlorine experiments are being repeated, with continuing support by the EPA, and complete chlorine data should be available soon in reports to that agency.

ASSOCIATION OF VIRUS WITH CLAY PARTICLES.

No extensive work was done with clay but we did see clear evidence of association of purified reovirus particles with particles of bentonite when they are examined by EM. The conditions were these: Bentonite (Volclay, ACCOFLOC-350-American Colloid Co.), 0.2 grams were put into 100 ml. of 0.005 M phosphate buffer, pH 7. Thirty-five ml of this was centrifuged for ten minutes in one tube of the Beckman SW-27 rotor at 5000 rpm (mean centrifugal acceleration 3900 G). The supernatant fluid was diluted 5x with the same buffer and mixed with an equal volume of reo stock virus containing about 2.4×10^{10} virions per ml in the same buffer. This mixture stood 3-1/2 hours at room temperature then it was diluted 84x with the same buffer and centrifuged over again for 30 minutes at 20,000 rpm for particle count. Pictures (Figs. 44 and 45) show both clay particles and virus particles clearly associated with practically no virions sitting alone. The higher magnification picture (Fig. 45) shows the plate-like clay particles in better detail.

ECHO-1 VIRUS.

Difficulties were encountered with ECHO virus in getting sufficiently high concentrations of clean virus for EM work but late in the program we did achieve this. ECHO-1 virus was obtained from Dr. Mark Sobsey of the Department of Environmental Sciences and Engineering, UNC, and passed 3 times in HEp-2 cells. After purification as for polio, the particle count by the KA method was 4×10^{11} virions per ml. and the particles were well dispersed (Fig. 46). No inactivation experiments were done with this virus but a series was performed, like that with poliovirus, to observe its state of aggregation in several kinds of water. Table 5 shows that in general dispersed ECHO virus aggregates less than polio and aggregated ECHO virus tends to disperse better than polio. A picture of dialyzed but unpurified ECHO virus (Fig. 47) shows the virus particles and their involvement with tissue debris much the same as was seen with similarly prepared poliovirus (Figs. 35, 36 and 37).

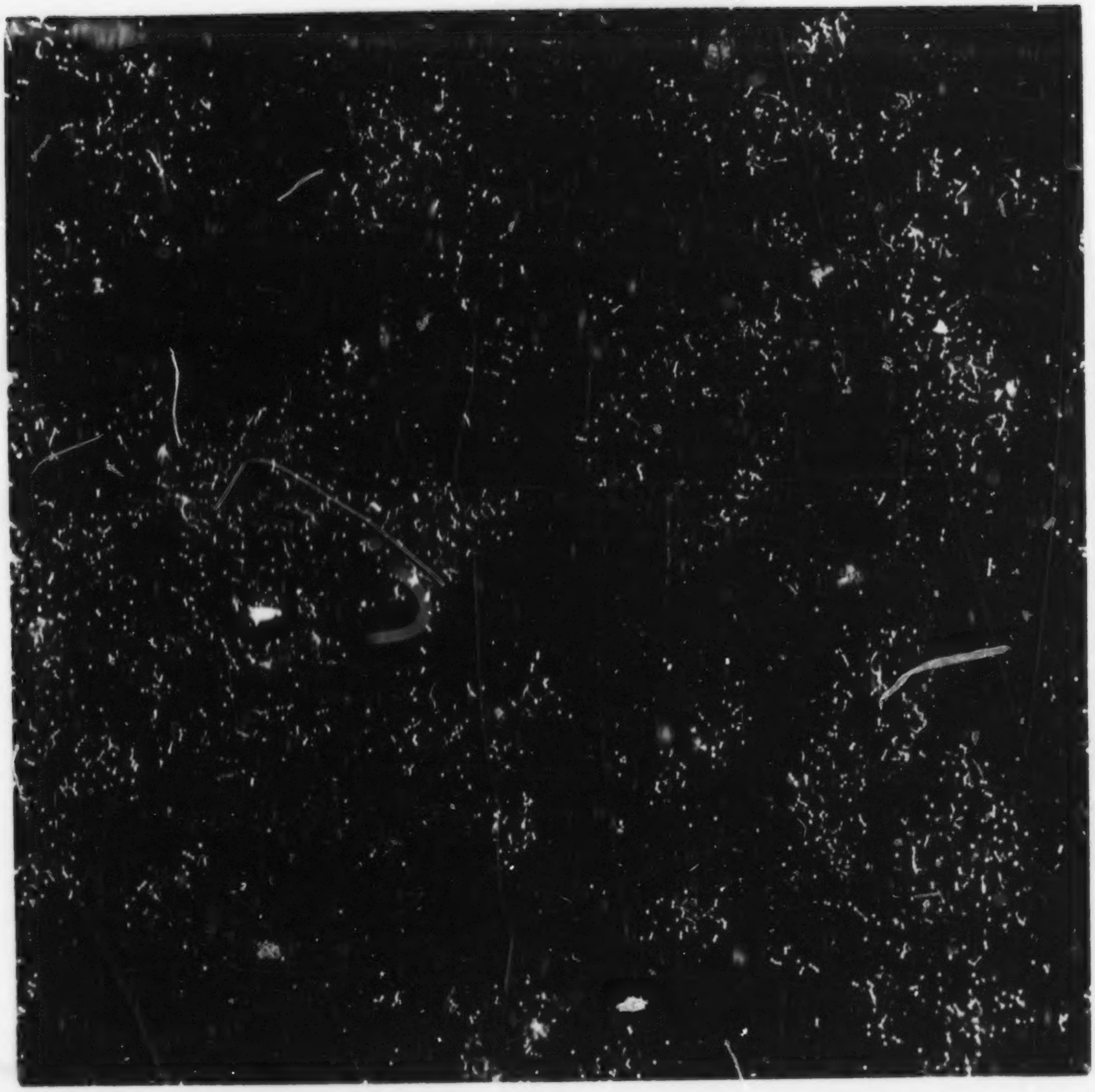

Fig. 44 Reovirus mixed with a suspension of bentonite clay then diluted and prepared for electron microscopy by the agar pseudoreplica method. Note the association of most of the virions with the clay particles. M = 14,000 X.

㉓ 24X

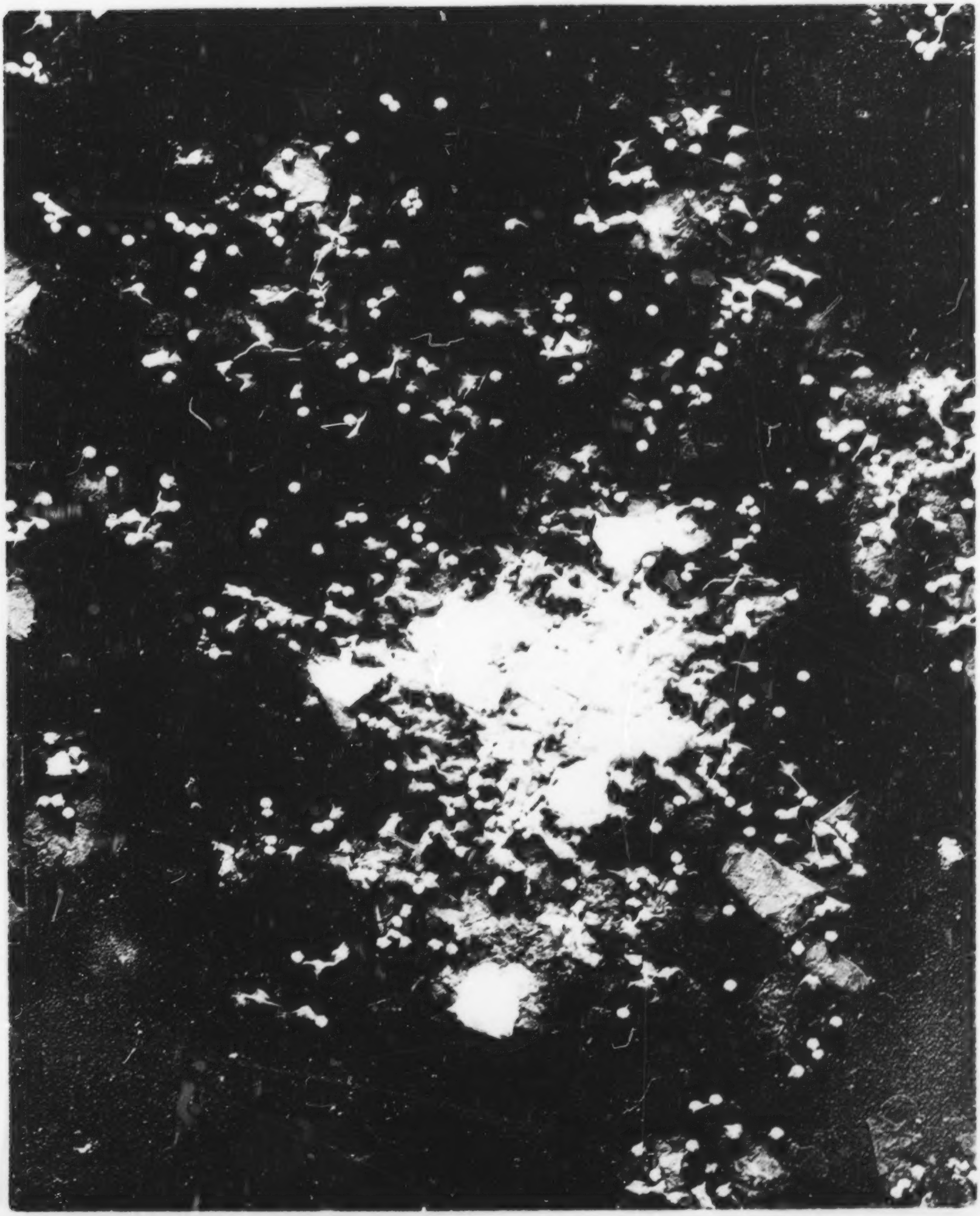

Fig. 45 Reovirus and bentonite clay particles at higher magnification (40,000 X).

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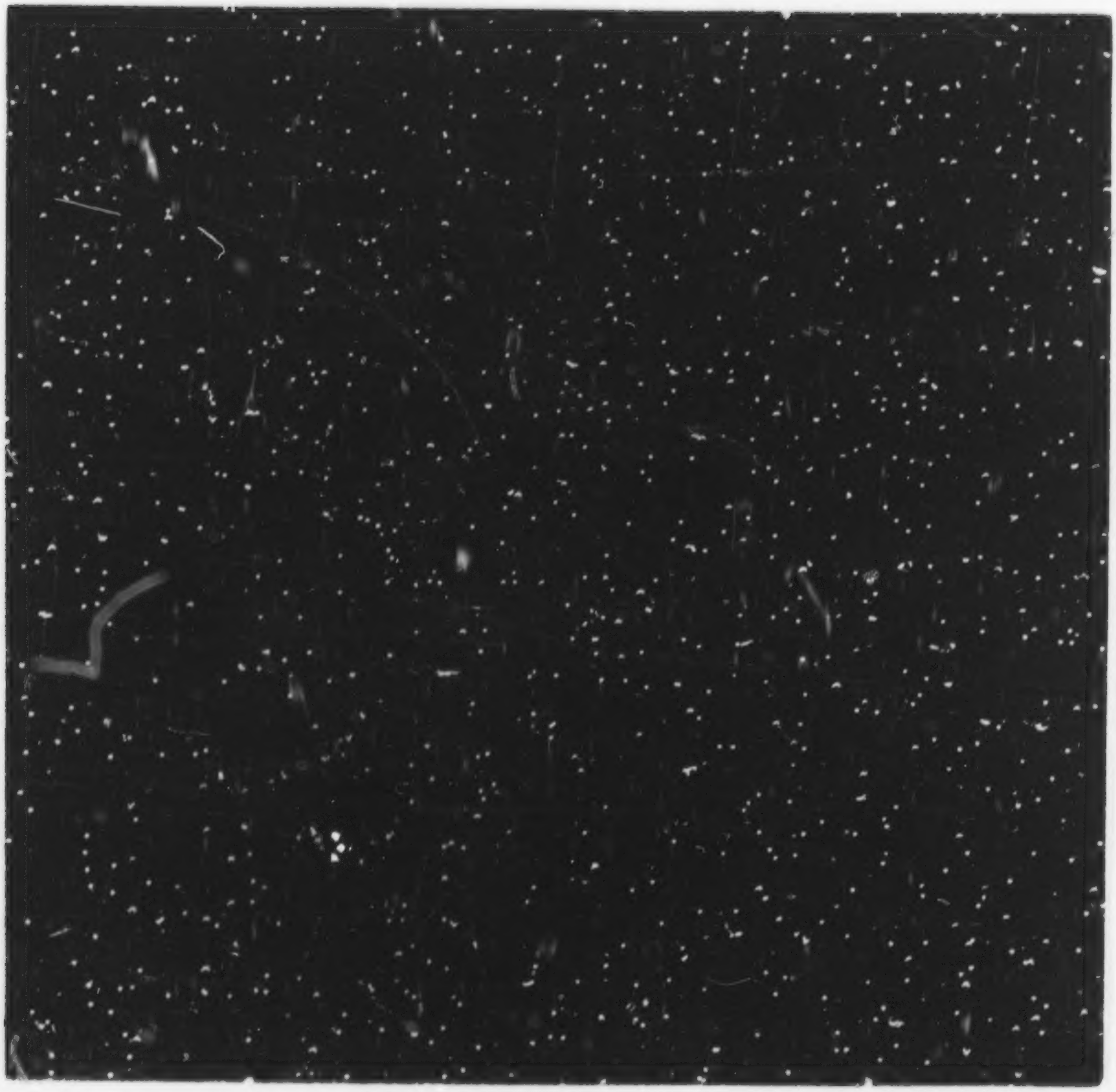

Fig. 46 ECHO-1 virus purified, prepared by the kinetic attachment method for display of the high degree of dispersion which is very similar to that obtained with poliovirus Fig 19. Mag. = 36,000X.

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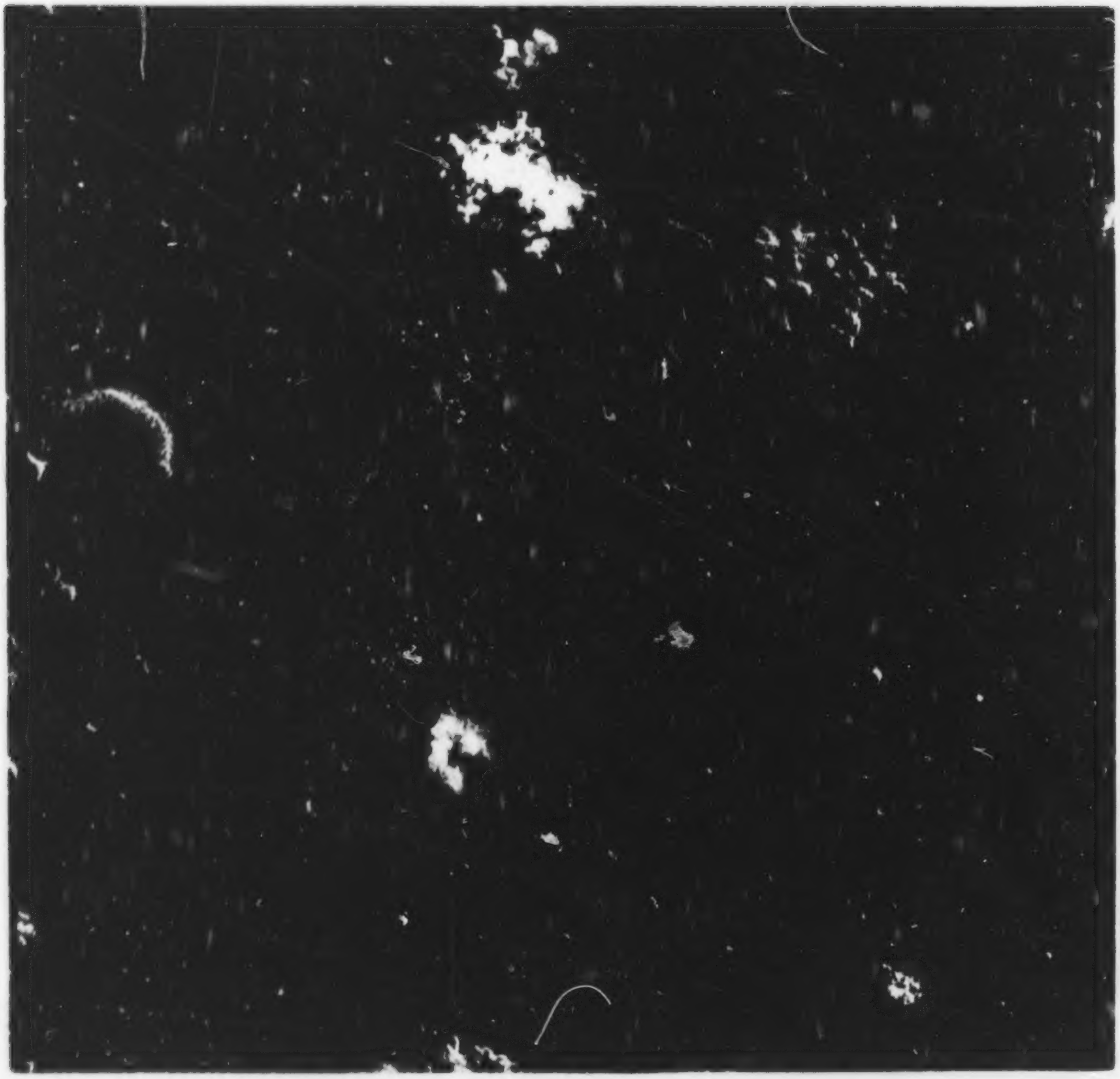

Fig. 47 ECHO-1 virus. Lysate of infected cells dialyzed over Amicon XM300 Membrane against water. M = 36,000X.

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㉕ 26X

DISCUSSION

Bromine is shown here to be a powerful agent for inactivating virus in water. Data are presented showing the individual effectiveness of HOBr, OBr^-, NBr_3, $NHBr_2$, and Br_2 in order that some rational estimate might be made of overall disinfective action in situations where more than one of these are present. Two viruses have been examined in some detail, polio and reo, but most of the inactivation data has been gathered with polio in the hope that its behavior is most likely to be representative of the large group of enteric viruses that may appear in polluted water.

A complication anticipated in the gathering of the above data was the state of aggregation of the virions when they encountered the bromine. So examination of the degree of aggregation and the conditions leading to aggregation of virus in water has been a major part of this work. In general, the logarithm of the number of survivors in the presence of a disinfecting agent is expected to decline linearly with time of exposure if the concentration of the active agent does not decrease in the process. However, this evidence of first-order kinetics is often not achieved in the laboratory. The more common experience is a curve indicating a decreasing reaction rate as disinfection proceeds. This condition has often been attributed to virion aggregation (1) but usually nothing has been done about it. This is a report of some things that have been done about it.

Electron microscopy, as it is usually done, is directed to attain maximum resolving power and the high magnification that goes with it. But our purpose is not to examine the ultrastructure of the virus but rather to see the virions in sufficient numbers to make an objective statistical statement of their state of aggregation. For this, the usual methods of preparing colloidal suspensions for EM study of their particles are quite unsatisfactory because they involve drying to deposit the particles on the surface to be micrographed. The forces of surface tension are so unpredictable during the last bit of drying that any particle aggregation seen in such preparations is quite likely to be an artifact of preparation. The basic problem is simply to produce a two-dimensional display of the particles from a three-dimensional liquid suspension without introducing any change in the size of the clumps or their frequency. Our two methods achieve a close approximation to these requirements by attaching the virions to the receiving surface before any drying takes place. In the SD method this is done by centrifugation and in the KA method by kinetic bombardment. In both cases every effort is made to remove all unattached virus before drying of the specimen. But even if there is no lateral displacement of particles from their original contact points there remains a predictable probability of one single particle or group falling upon another. This, of course, can be minimized experimentally by dilution and calculated for dilute preparations from basic mechanics (30). Having produced an acceptable preparation of the virions for the EM one must use the lowest practically attainable magnification in taking count pictures in order that the greatest possible number be seen in the picture. This sets the maximum sensitivity and therefore the most dilute suspension that will yield quantitative data and the minimum number of pictures required to get it. We have found for routine work with reovirus (70 nm dia.) that electron magnification of 2000 to 2500 is suitable and for the smaller polio and echo viruses 5000X is necessary. These pictures can be conveniently analyzed by projecting them (they are 2 inches square, 5 per glass 2" x 10" negative) on a flat white surface 4 feet square divided into 25 squares. In this way we have made direct observation of the physical state of stock preparations of all three viruses and of changes that occur when dilutions are made with changes in pH and/or in ionic strength. Particle counts are very convenient also for locating an invisible virus band in a density gradient when plaque titration would be laborious and require much more time (Figs. 19 and 20).

In order to get the data described in the first paragraph of the discussion it was necessary to establish exactly reproducible conditions particularly with regard to the physical state of the starting virus. Inasmuch as we know of no way to reproduce or even maintain virus preparations with any particular degree of aggregation we decided that meaningful comparisons of viruses and their reactions with different disinfecting agents can be made only with preparations containing no clumps at all. Furthermore the act of dilution of such virus stock suspensions into the water containing the disinfectant must not induce aggregation. These things have been monitored by electron microscopy with the result that essentially monodisperse stocks of virus have been produced and unexpected aggregation caused by dilution in fresh (low ionic strength) water detected.

Examination of the data will show that the reaction rates are very fast with bromine. With HOBr, for instance, reovirus lost infectivity at the rate of 3 $\log_{10}$ units per second (Fig. 15) at 2C and a concentration of 2.9 μM. Even the more resistant polio virus was about 98% inactivated in 16 seconds under the same conditions (Figs. 20 and 21). So the dynamic, turbulent flowing stream type of experiment (Fig. 2) was needed to produce samples of virus for titration after an exposure of as little as 0.5 seconds. This required design and construction of apparatus to provide for turbulent flow of bromine water of known temperature and velocity through tubing and fixtures that have negligible bromine demand. Virus must be injected at a known rate for a known period of time and samples withdrawn and quenched with sodium thiosulphate in times that are negligibly short compared with the minimum transit time of 0.5 seconds. These stringent conditions have apparently been met as can be seen in Fig. 15 where virus samples taken at a series of 0.5 second intervals showed a log-linear decline in titer with time. It must be emphasized that laminar flow must not occur in any part of this experiment because virus particles near the walls of the tubing will move more slowly than those in the center. This is avoided if the Reynolds Number is kept above the critical value to main turbulence.

At the beginning of this work we sought a mathematical relationship (33,34) between the observed degree of aggregation of a virus particle suspension and the curve of Log Survival Ratio of PFU plotted against time. Data of the kind shown plotted in Fig. 15 can be employed for this purpose (35).

If about one PFU in 10,000 of the starting titer were an aggregate large enough to protect one potential plaque forming virion from destruction by the bromine, this would be enough to account for the persistent infectivity of reovirus seen in the experiments of Fig. 13. If such an aggregate consisted only of virions (no extraneous material) there would seem to be no way that such small groups as 2, 3 or 4 could conspire to protect even one of their number from attack by the large number of small bromine molecules or ions. Effective protection would not seem possible with much less than a complete monolayer of particles surrounding the protected one and this can happen with spheres of equal size only if there are at least 17 in the clump. Clumps of this size would be easy to see with the EM, but at a frequency of 10^{-4} they might easily go unnoticed. The picture did show that no groups larger than 7 virions were present at a frequency greater than 1:1000 and such a cluster could not quite cover one half of the surface of one. They could not afford it much protection. How, then, can such a survival curve as that shown for the slightly aggregated reo virus of Fig. 15 come about?

The upper curve of Fig. 15 has been repeated twice here for demonstration of two different qualitative analyses. If all the virions are alike in this mixture of singles and aggregates and the probability that any single particle will make a plaque is E then the starting titer when t = 0 (before bromine treatment begins) must be given by:

$$T_o = E\ (N_1 + C_2N_2 + C_3N_3 + \ldots\ldots C_MN_M) \qquad \text{Eq 1.}$$

where N_1, N_2, N_3, etc. are the numbers of singles, pairs, triplets, etc., where M is the maximum clump size present in significant numbers and C_2, C_3, C_4, etc. are factors, the values of which we will now seek.

If we were dealing with perfect virus (E=1.00) then each single, each pair, each triplet, etc., would make just one plaque and no more; C_2=C_3=C_4 etc. = 1. But the reovirus used here had about 40 times as many particles like those of Fig. 6 than the plaques it produced. This is saying that on the average only one virion in about 40 succeeds in making a plaque. If random small groups, pairs, triplets, etc., are made from such singles there is only 1:40 chance of finding 2 of the plaque-forming singles in one such pair so there will be little loss through redundency and the probability of plaque formation by a pair, triplet, etc., would seem to be very nearly 2X, 3X, etc. greater than that for single particles. Briefly, if the plaquing efficiency of the virus is low (as this is) the probability of plaque formation by a small clump of i particles would be approximately i times that for any one particle and equation 1 above can be approximated by:

$$T_o = E\ (N_1 + 2N_2 + 3N_3 + \ldots\ MN_M) \qquad \text{Eq 2.}$$

If this is the starting titer, its logarithm will be subtracted from that of each survival titer so the E, whatever its value, does not appear in the difference that is the logarithm of the survival ratio which is plotted on the disinfection graph. The numbers N_1, N_2, N_3, etc. are supplied by the electron microscope (Fig. 14) and the logarithms of the corresponding set N_1, $2N_2$, $3N_3$, etc., up to MN_M are plotted as shown (Fig. 48) along the vertical zero time axis. The set used to plot the dotted line of Fig. 48 as it appeared in the original publication (35) was simply N_1, N_2, N_3, etc. The EM data do not go much beyond 1 group per 1000 particles so this analysis probably should not include groups of greater than 7 particles (m=7) and the approximation to the experimental curve should be accurate until single particles have been reduced 99.9% by the bromine.

Having gotten the starting points for each group size in the mixture, one can draw immediately the best straight line through the data points for inactivation of single particles. This would indicate

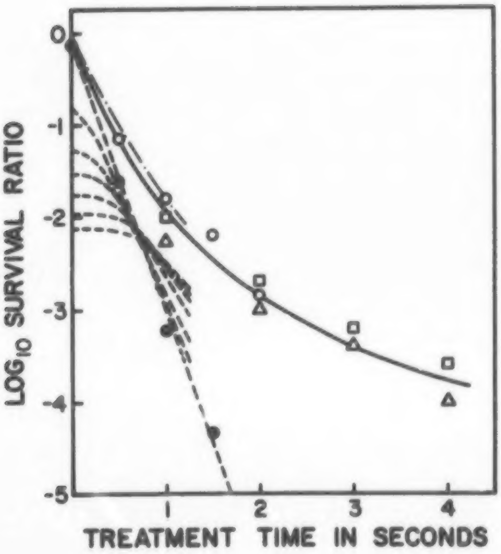

Fig. 48 Theoretical approximation to the observed bromine disinfection curve of Fig. 15 using the measured rate for single virions, the numbers of pairs, triplets, etc., known from electron micrographs and assuming Poisson multiple-hit kinetics. The curve is the sum of the contributions from each of the seven lines, one for each group size from 1 to 7.

a titer reduction from T_0 to T_m after a mean dose m of bromine; $T_m = T_0 e^{-m}$. If one assumes that the survival of a group of i particles is predicted by the multi-hit target concept then the Poisson limit gives

$$T_m = E\left(1+m+\frac{m^2}{2!}+\frac{m^3}{3!}\ \cdots\ \frac{m^{(i-1)}}{(i-1)!}\right)e^{-m}$$

and the survival titer of the whole mixture will be

$$T_m = E\left[N_1+(1+m)N_2+\left(1+m+\frac{m^2}{2!}\right)N_3\cdots\right]e^{-m} \qquad \text{——— Eq 3.}$$

So now we can use the straight single's line to determine m in terms of t, then calculate from equation 3 and draw, as on Fig. 48, the predicted survival curve beginning at the proper point indicated above for pairs, $T_{2m} = E(1+m)e^{-m}$

for triplets $T_{3m} = E\left(1+m+\frac{m^2}{2!}\right)e^{-m}$ etc. making 7 lines in all.

Now, for any chosen time the predicted survival titer for the mixture will be given by the sum of the antilogs of the seven intersections with this vertical time line. The locus of the logarithms of these sums forms the curve drawn for comparison with the observed data. The fit of the predicted curve to the data is quite good.

Another, perhaps equally good fit to the experimental data can be obtained by drawing straight lines (Fig. 49) from the same set of starting points that were used in Fig. 48. The slopes of these lines (all except the single's line that has been determined by experiment) can be found by trial until the sums of the contributions from all the significant group sizes makes a total the $\log_{10}$ of which falls on or near the experimental curve for all times within the range tested. By means of such graphical experimenting it is not difficult to achieve a good fit as may be seen and each of the lines can be expressed as

$T_{it} = T_{i_0} e^{-K_i t}$ where K_i is the reaction constant for groups of i particles. Unlike the multi-hit Poisson concept of virus disinfection which gives the exact curve for each group size, this method shows only that if all the reactions are first order (straight lines) a set of slopes can be found, consistent with the known numbers of each aggregate size present, to account quite well for the observed results.

Both of these graphical analyses provide a tolerable fit to the experimental disinfection curve down to the point where the survival ratio of single particles is 10^{-3} and that of the aggregated preparation is 10^{-2}. Beyond this point the EM does not supply the needed accurate

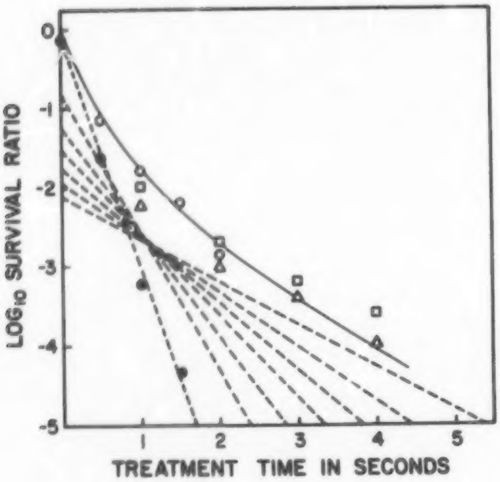

Fig. 49 Theoretical approximation to the observed bromine disinfection curve of Fig. 15 using the same data as in Fig. 48 but assuming each group size decays at its own constant rate. These rates, slopes of the six lines representing group sizes from 2 to 7, have been drawn from known starting points by trial so that the sum of the survival titers they represent will make the best fit to the actual data points shown.

data on the frequency of clumps containing 8 or more virions. One is tempted to extrapolate the observed linear relationship between Log N_i and log i to provide a rational basis for extending the predicted disinfection curve but this must be done with care because of increasing closeness of the lines (Fig. 49) or curves (Fig. 48) for each group size. This can be seen on either graph and it suggests a limit to useful extrapolation. While this might discourage those who seek a simple method of predicting the course of disinfection beyond any given experimental limit, the above exercise has provided a means of answering a significant question about the mechanism of survival through aggregation.

The question is whether small clumps of virus, even pairs, are more resistant to bromine action than single particles or whether, after exposure to bromine, the virions of a pair or small clump can help each other in the act of plaque formation that would not occur if they were acting separately. If we consider the point at which the PFU titer of single particles has dropped by a factor of 10^{-3}, some reason must be found for the observed survival titer of the aggregated virus which is 10 times greater. There are not enough large aggregates present to account for this and only in large aggregates can one expect protection of one or a few of their number from contact with the bromine. Even small aggregates are not frequent enough (Fig. 14) unless they survive much better than single particles. By either of the graphs it is clear that when the mean treatment (m=7) has reduced singles to e^{-7} which is just about 10^{-3} of their original titer, pairs for example must be 8 to 10 times more resistant than singles in order to contribute enough to the total observed titer. This author thinks it unlikely that two virions can contrive to protect each other to this extent, therefore their demonstrated survival must be a synergistic effect resulting from complementation in plaque formation. This is probably the dominant mechanism of reovirus PFU survival down to 10^{-3} and perhaps farther but Fig. 13 showed that the large aggregates were dominant in survival at about the 10^{-4} level. So it seems that both modes of survival are operating with aggregated reovirus exposed to bromine.

It can be seen that nice orderly aggregation like that discussed above can be treated analytically with some success but the prognosis for accuracy of any extrapolation far enough to include the large aggregates of virus that are probably present in naturally polluted water is not good. It may be better at some future time but for now it seemed better to note it down and just get on with finding out what conditions lead to virion aggregation in water, how fast it occurs and how much might be expected in natural water.

In general there are two ways to demonstrate virion aggregation in very dilute suspensions. Filters selective enough to separate single particles from aggregates are available from several manufacturers and they are easy to use but unfortunately their selectivity is not determined by particle size only. They all involve the passage of virus particles in close proximity to the filter structure to which they tend to be attracted more or less (at different values of pH and ionic strength) by electrical forces and influenced by the presence of even trace amounts of soluble proteins and other colloidal "stabilizers". This greatly restricts their value in quantitative work of this kind so we have turned to the ultracentrifuge in which the sedimentation velocities of single virions are known to be much (about) 40% less than the smallest aggregates (pairs of spherical particles) and they are very little affected by particle concentration, pH or ionic strength of the suspending fluid. Two practical methods for aggregation analysis by sedimentation velocity have been developed here. The SPA method gives just a single particle approximation (% of total plaque titer contributed by single particles). This provides a single statistic characterizing an aggregation but it is a useful one and one that can be compared directly with the theoretical prediction of von Smoluchowski (36) for rapid coagulation of colloidal particles in general. This will be discussed later.

More detailed information on the aggregated state of a virus preparation has been obtained in a second variation of the sedimentation velocity experiment that we have called the Red, White and Blue analysis. This involves the partition of singles and aggregates from a virus sample in a centrifuge tube containing a 3 step density gradient. Now, instead of titrating just the top fraction, as in the SPA test, all three are titrated and the percentage of titer in each gives a useful arbitrary separation of the size spectrum into 3 parts. Much of the comparative data on virus aggregation or aggregate dispersion in various waters was gained in this way.

Having shown that clumps of virions are much more resistant to bromine than single virions and that clumps of the sensitive reovirus may survive bromine doses that destroy single particles of the much more resistant poliovirus, it becomes apparent that the sanitary engineer must be more concerned with aggregated virus than perfectly dispersed virus. But what kind of aggregates will he be dealing with and how do they come about?

Our results show that poliovirus, released by freezing and thawing a few infected cells directly in lake water, remains largely aggregated. Most of the infectivity resides in clumps of 10 or more virions. This is probably because poliovirus is generated in large cytoplasmic clumps or crystals that are not dispersed in fresh water (in sea water they break up completely). These we might call original aggregates and it is probably these that will be the survivors of insufficient water treatment.

But, at least in the laboratory, there are other kinds of aggregates too. A ten fold dilution of buffered salt suspensions of single virions in water often causes copious aggregation which does not break up with further water dilution. Addition of salt (further dilution with physiological saline, for instance) will disperse such aggregates of poliovirus but not of reovirus. Thus it is possible to dilute a stock poliovirus preparation in water where aggregates occur then make a further dilution series for plaque titration (using PBS, of course, because this is standard procedure) and the dilution that reaches the tissue culture cells will contain only single particles. This is one thing we have found that may account for some puzzling results of disinfection experiments with viruses. Apparently all viruses are not alike in this respect. Water-induced aggregates of reovirus persist on further dilution with PBS.

Both polio and reoviruses aggregate at pH values below seven; the lower the pH the faster they aggregate but these acid-induced aggregates are quite completely dispersed when they are returned to pH 7. Treatment of virus with bromine in acid (pH 5) buffers, which was necessary with Br_2 for example, would always involve aggregation were it not for the fact that addition of neutral salts (NaCl or $MgCl_2$) can prevent it. Magnesium chloride is much more effective than the sodium salt. This effect is qualitatively the same as it is on certain proteins such as the globulins. It is not to be confused with the salting out effects that occur at much higher concentrations of proteins.

We have observed the aggregation of poliovirus when stock concentrates are diluted 10 fold in water and also found that a 1000 fold one-step dilution of the same stock virus in water did not aggregate. It is quite likely that the virions in both of these dilutions are unstable. Given sufficient time, the 1000 fold dilution would probably aggregate to the same degree that is reached quickly at 10 fold dilution. Such apparent or quasi stable conditions must be encountered frequently in experimentation with viruses in water in which there is little or no salt, particularly under acid conditions. Modern textbooks such as those of Hartman (37) and Kruyt (38) quote the original works of von Smolukowski (36) on a mathematical theory for the coagulation kinetics of colloidal solutions. Tests of the theory were made at that time with gold sols and such organic colloids as those of mastic and gamboge that were used so extensively by Perrin (39) in his basic experiments on Brownian motion. No tests of this long-standing theory have been made, as far as we know, with viruses. There seems to be no apriori reason why viruses some of which are in the size range employed by Perrin, should not obey the same laws of coagulation. Furthermore the uniformity of particle size of viruses is a highly desirable feature and our quantitative methods of electron microscopy permit an accurate appraisal of the nature and degree of the aggregation that occurs. So we have calculated from the theoretical equation of von Smolukouski the expected numbers of single virus particles remaining in an originally dispersed suspension that have not yet aggregated and plotted these as a function of time for several levels of concentration (Top line of Fig. 50). This time scale of the graph is the same as that used in our SPA experiments with both reo and polio viruses. It can be seen by comparing the observed data (Figs. 28,32) with these theoretical predictions for maximum possible reaction rates (every collision sticks) that the shapes of the curves are similar and at pH 3 the initial rates are comparable. At pH 3 it appears that both reo and poliovirus do begin to aggregate as though only Brownian motion is controlling. However, the observed data show a general tendency to level off more quickly and continue to show more single particles than predicted. This could be due to some residual error in the experiment. If so, we do not know what it is. Both viruses aggregating at pH 5 or in water proceed much slower than predicted by Fig. 50 indicating that only a small fraction of the collisions actually result in aggregates.

The theory of von Smolukowski predicts also the number of pairs, triplets etc. that should be present as a function of time when a suspension of single particles aggregates at maximum rate. We have calculated from his equations and plotted on a log scale a set of such curves (Fig. 50) extending over a time period equal to 3 times the half life or coagulation time. It will be apparent if one takes a set of values from the several curves at any chosen time and plots them as ordinates against the logarithm of group size as has been done (Fig. 51) for one half life, that the line is a curve with increasing slope as group size increases from 1 to 6. Herein lies a readily demonstrated difference between these predictions and observed results with viruses. With reovirus we have seen (Fig. 14) in similar plots made from electron microscope data that the line is straight. With vaccinia virus it is straight (26,27) and unpublished results in this laboratory show that with polystyrene latex particles (0.24 μ from

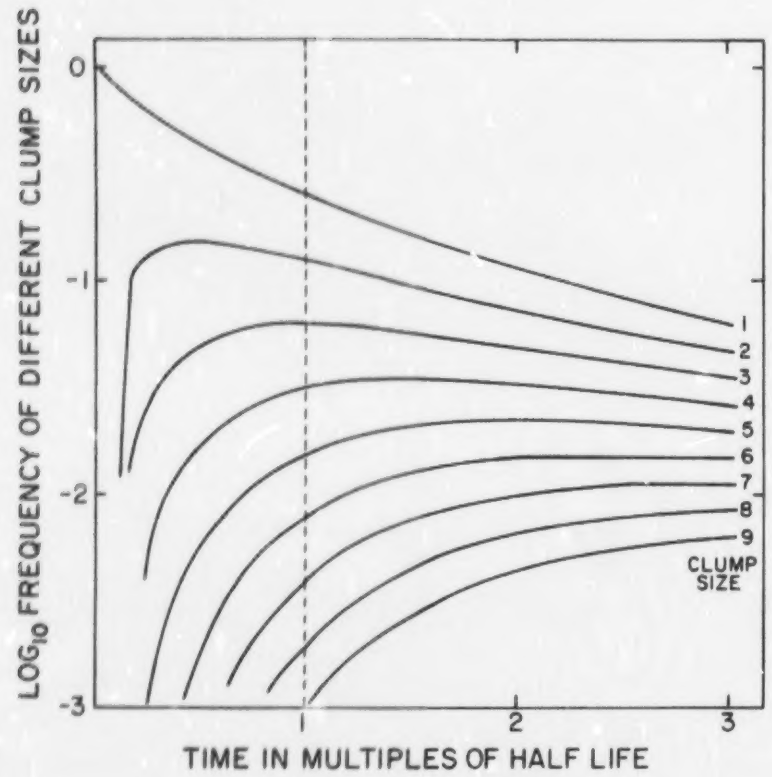

Fig. 50 Theoretical relative frequency of clumps containing one through nine particles in a suspension subject to rapid coagulation. The half life (time scale) is the time required for the total of singles plus clumps to be reduced to half the original number which were all singles at the start. These curves were calculated from the equations of von Smolukowski (20).

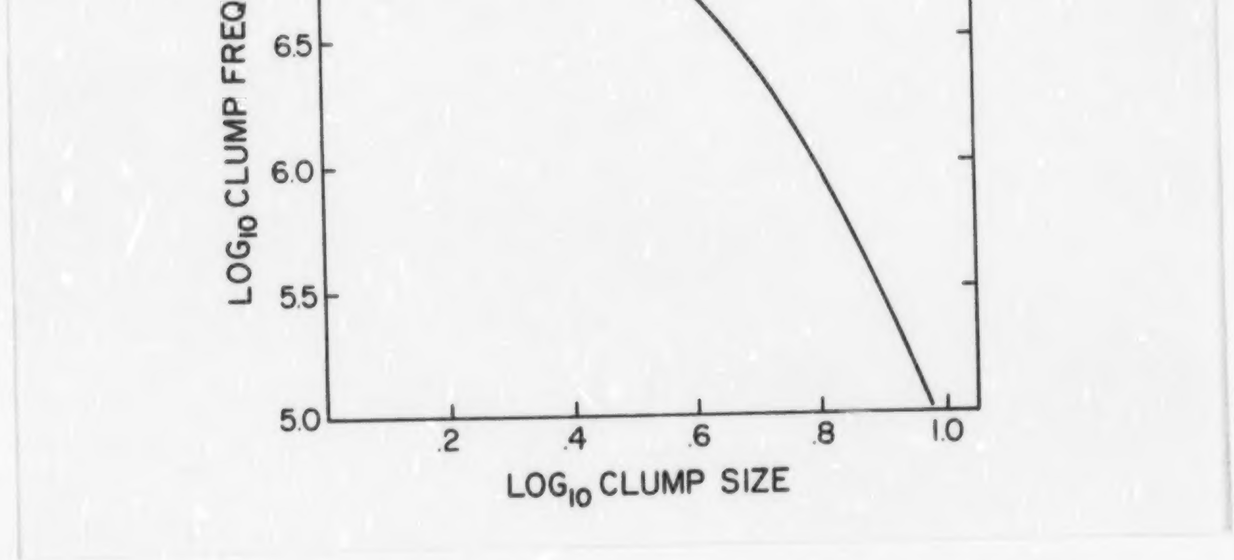

Fig. 51 Log-Log frequency vs clump size can be predicted from the theory of rapid coagulation of von Smolukowski by drawing a vertical line at a chosen time through the family of curves of Fig. 50. These can be used to generate this curve which is quite different from the straight lines (Fig. 5 and 14) obtained from the electron microscope with reovirus and the fewer but similar data on poliovirus in Table I.

Dow Chemical Co.) as well as with acrylic resin particles (0.5 μ from Colab Laboratories, Inc.) the line is straight. Take, for example, Fig. 51. The time chosen for this set of points was the coagulation time or "half life", the time when the total count of single particles plus clumps has fallen to half the original value. At this time the number of singles will have dropped to one fourth. The curve is unmistakable, when compared with the straight line data obtained from electron micrographs and the discrepancy is roughly 4 fold for clumps of 6, a difference well beyond the limit of experimental error. There are fewer aggregates seen than predicted and the difference increases with group size.

While we have labored here to show that there are significant differences, both in the rate of disappearances of single virions and in the frequency distribution of group sizes from those predicted by the theory of rapid colloidal flocculation the fact remains that von Smolukowski's theory is still a good guide, the best we have for virus behavior. It does predict approximately, within the limitation described above, the kinetics of virus aggregation as we have observed it.

SUMMARY

Bromine has been shown here to be a powerful agent for the inactivation of virus in water. Of the various forms in which bromine can exist in water, OBr^- reacts most rapidly with poliovirus reducing its plaque-forming infectivity about 3 $\log_{10}$ units per second at a concentration of 9.6 µM at 2C. This was at pH 11. At pH 5 with Br_2 the rate of inactivation was 0.8 $\log_{10}$ units per second. With HOBr and the bromamines (NBr_3 and $NHBr_2$) the corresponding rates were 0.12, 0.035 and 0.004 $\log_{10}$ units per second, all at pH 7. So the three forms of greatest activity are OBr^-, Br_2, and HOBr, in that order. These reaction rates have been determined with preparations of purified virus that were well dispersed.

Reovirus is much more sensitive to bromine (HOBr) than poliovirus. At a concentration of 2.9 µM it lost PFU titer at 2C at the rate of 3 $\log_{10}$ units per second when it was, like the poliovirus above, well dispersed. But when aggregation was present reovirus survived bromine treatment that destroyed well dispersed poliovirus. Aggregated preparations of both viruses reacted with HOBr in the same way, that is, with a continuously decreasing reaction rate characteristic of a mixture of infectious units with different degree of sensitivity. Clearly the most resistant units are the larger aggregates but we have not established the mechanism of survival. It might be either a protection effect during exposure to the bromine or the result of complementation resulting in more efficient plaque formation by clumps of damaged virions which may be individually incapable of infecting a cell.

Special apparatus was designed and built to make the measurements of surviving virus infectivity after bromine-exposure times as short as 1/2 second. This apparatus has performed well in over 200 experiments and we recommend it to others who will work in this field.

We have found the dependence of inactivating rate on concentration of bromine at temperatures near freezing is generally not linear. This is particularly noticable with NBr_3 and HOBr. Increasing the concentration increases the reaction rate but not proportionally. At 10°C and at 20°C this effect is much less and rates are essentially proportional to concentration.

Doubtless the most significant findings reported here are those pertaining to aggregation of the virus particles and the effect of this aggregation on the disinfection rate achieved by the bromine. All of the results mentioned above were obtained with virus that was carefully prepared and checked by electron microscopy to insure the absence of clumps of virions. We believe that meaningful comparisons of disinfecting rates cannot be obtained in any other way. However, we have shown that virus particles are generally aggregated in the crude (unpurified) state and that there are several, perhaps many, conditions that promote reaggregation of dispersed virus. Such aggregation always increases bromine resistance. We have demonstrated this aggregation first by electron microscopy but we have also developed two relatively simple experimental techniques for assay of aggregation that are applicable at very low virus concentrations. With these we have observed that dilution of purified stock virus into water often causes copious virion aggregation which does not disperse with continued water dilution. Furthermore the release of virus from infected cells directly into water tends to leave much of the virus in aggregates that persist. This is in water, not the usually buffered isotonic salt solutions usually used in virus laboratories.

Acid conditions promote progressive aggregation of both reo and polio viruses in water and this would have made it impossible to make Br_2 experiments (pH5) with dispersed virus were it not for the discovery that addition of salts to the water may prevent aggregation. Some data are presented on the kind and quantity of salts that are effective in this respect.

Data are presented showing the fate of aggregates of virus particles when they are put into water of several kinds including sea water. Dispersed virus was put into this same series of waters too, in order to observe any tendency to produce aggregation. Aggregates tend to persist in fresh water; sea water appears to be able to disperse them. But the above pertains largely to pure aggregates, just clumps of virions. Virus appears to clump in finished treated drinking water after lime and alum treatment. This is probably due to complex formation (virions attached to insoluble salt particles). Attachment of reovirus particles to clay particles (Bentonite) is shown here by electron microscopy.

We have found little or no aggregation of purified poliovirus at pH values above 7 but under suitable acid conditions where aggregation is rapid, the rate of clump formation has been followed by electron microscopy as well as by measuring the fraction of single particles that remain after a period of time. These data show that the kinetics of the aggregation of these virus particles are second order, quite similar to those predicted by the theory of rapid coagulation of colloids. One exception to this general approximation that has been observed is that the frequency of clumps of larger size is a little greater than the theory predicts.

ECHO-1 virus, in a few experiments near the end of this work, appears to be slightly less subject to aggregation than the physically similar polio virus.

It is quite clear that the bromine resistance of aggregates of virus particles rather than the resistance of single virions, is the significant quantity that must guide sanitary engineers who would disinfect water containing virus.

RECOMMENDATIONS

At the end of this period of research investigation it is clear that more needs doing:

1. We have examined the bromine sensitivity of polio virus in some detail, and reovirus in somewhat less detail. They are very different and no one can predict, from these two alone, how other enteric viruses will behave. Other viruses must get equal or more attention.

2. We have solved some of the aggregation problems peculiar to these two viruses in order to get comparative bromine-resistance data on single-particle suspensions. This means that our reaction rates are not exactly comparable with chlorine, iodine or ozone data obtained by others who have used aggregation virus. So, similar experiments should be made with these and perhaps other active agents.

3. We have not examined the higher temperature range (25-30^{o}C) like midsummer in Texas. This should be done too.

4. We have uncovered a few facts about simple virion-to-virion aggregation that are vital to work in fresh water (low salt). These phenomena will strongly influence both disinfection resistance and the titration of surviving infection by viruses in water. More must be learned about the aggregating behavior of these two viruses and that of others as well.

5. We have struggled and wrought nothing much about the possibility that damaged virions may subsequently aggregate and thereby increase their infectivity through complementation (MR). We are continuing the effort and others might do well to try.

6. Our data on aggregation of polio (and a little on ECHO) in natural waters came by way of a suggestion by Dr. Schaub (40). Our SPA and RWB techniques, developed for this purpose, have revealed some practical facts but they have merely scratched the surface of a great body of ignorance that can only be dispelled by continued diligence and further work in this area with several viruses.

7. Complex formation, adsoption of single virion or clumps of virions to foreign matter, either organic or inorganic, must certainly be examined in more detail than has been done by anyone up to now. Methods such as we are using would be admirably suited to such work and hopefully they would supply some definitive data on the influence of such complexing on resistance to disinfection as well as on efficiency of infection by the survivors of disinfecting agents.

8. Accurate determinations of the isoelectric points of the enteric viruses are very few in the literature. These are needed if one is to build a rational concept of the mechanism of the virion aggregation that occurs at acid pH.

9. The observed failure of the disinfection rate of HOBr on poliovirus to increase in proportion to concentration is a matter of great practical importance. The consequences of this effect must be noted and the mechanism sought.

10. There is still no clear concept of the mechanism by which each of the species of bromine reacts with and destroys the infectivity of animal viruses. There is much to be done.

TABLE 1

Distribution of group sizes in a typical poliovirus preparation.

Group Size	Group Frequency per area*	Standard Deviation	Total Groups Counted
1	124	15	2480
2	4.5	2.1	89
3	0.35	NC[xx]	7
4	0.05	NC	1

* Twenty areas counted. This column gives the average per area.

xx Not calculated.

TABLE 2

Ionic Inhibition of Poliovirus and
Reovirus Aggregation at Low pH.

	pH 6		pH 5		pH 3	
	Na^+	Mg^{++}	Na^+	Mg^{++}	Na^+	Mg^{++}
Poliovirus	0.1M	0.01M	0.2M	0.25M	2.5M	0.25M
Reovirus	NA*	NA	0.6M	0.25M	>1.0M	0.25M

*NA: Not applicable since reovirus did not aggregate at pH6.

TABLE 3

EFFECT OF DIFFERENT WATERS ON THE PHYSICAL STATE OF POLIOVIRUS

3 Categories of Aggregation

White (W) Essentially all singles
Blue (B) Singles and small aggregates
Red (R) Large aggregates
Data are % of PFU found in each category

INPUT VIRUS CONDITION

WATER	DISPERSED						AGGREGATED					
	1 min			1 hr.			1 min			1 hr		
University Lake	W	B	R	W	B	R	W	B	R	W	B	R
Raw	57	31	12	58	33	9	8	22	70	16	30	54
Clarified	54	39	7	51	45	4	15	34	51	14	43	43
Filter plant-Finished	6	51	43	6	50	44	10	19	71	7	19	74
Sewage Plant Secondary effluent												
Raw	73	20	7	76	15	9	28	31	41	45	36	19
Clarified	63	33	4	60	34	6	61	32	7	69	29	2
Controls												
PBS	60	37	3	55	44	1	54	42	4	60	37	3
Distilled H_2O	36	62	2	32	62	6	7	21	72	5	26	69
Dist.H_2O+Ca^{++}	65	35	0	75	25	0						
Dist.H_2O+Ca^{++}+AL^{+++}	17	6	77	10	7	83						

One drop (Ca 0.03 ml) of stock virus was mixed into 200 ml of test water giving a titer of about 10^4 PFU/ml. Samples were withdrawn one minute and one hour later, separated into three parts in the centrifuge and titrated on monolayer cell cultures. Well dispersed virus will be about evenly divided between the White and Blue with 0 to 3% Red.

TABLE 4

PHYSICAL STATE OF POLIOVIRUS RELEASED BY FREEZING AND THAWING A FEW INFECTED CELLS IN DIFFERENT WATERS

(Final titer ca 10^4 PFU/ml)

		Filter plant		Sea water		Secondary sewage plant effluent		Controls	
FRACTION	Raw	Clarified	Finished	Straight	Clarified	Raw	Clarified	Distilled water	PBS
Singles (W)	25	33	9	51	63	30	36	56	56
Small Aggregates(B)	20	35	16	41	31	35	33	34	38
Large Aggregates(R)	55	32	75	8	6	35	31	10	6

Data are % of PFU found in each of the three categories of aggregation - see table 3.

TABLE 5

EFFECT OF DIFFERENT WATERS ON THE PHYSICAL STATE OF ECHO VIRUS

3 Categories of Aggregation

White (W) Essentially all singles
Blue (B) Singles and small aggregates
Red (R) Large aggregates
Data are % of PFU found in each category

	INPUT VIRUS CONDITION											
WATER	DISPERSED						AGGREGATED					
	1 min			1 hr.			1 min			1 hr		
University Lake	W	B	R	W	B	R	W	B	R	W	B	R
Raw	71	27	2	60	39	1	48	40	12	74	24	2
Clarified	52	46	2	61	37	2	32	60	8	34	64	2
Filter plant- Finished	55	42	3	58	40	2	53	45	2	44	54	2
Sewage Plant Secondary effluent												
Raw	72	26	2	79	19	2	53	44	3	68	29	3
Clarified	58	38	4	67	28	5	90	9	1	91	8	1
Controls												
PBS	59	40	1	53	46	1	71	26	3	74	24	2
Distilled H_2O	41	54	5	59	39	2	36	42	22	35	52	13

One drop (Ca 0.03 ml) of stock virus was mixed into 200 ml of test water giving a titer of about 10^4 PFU/ml. Samples were withdrawn one minute and one hour later, separated into three parts in the centrifuge and titrated on monolayer cell cultures. Well dispersed virus will be about evenly divided between the White and Blue with 0 to 3% Red.

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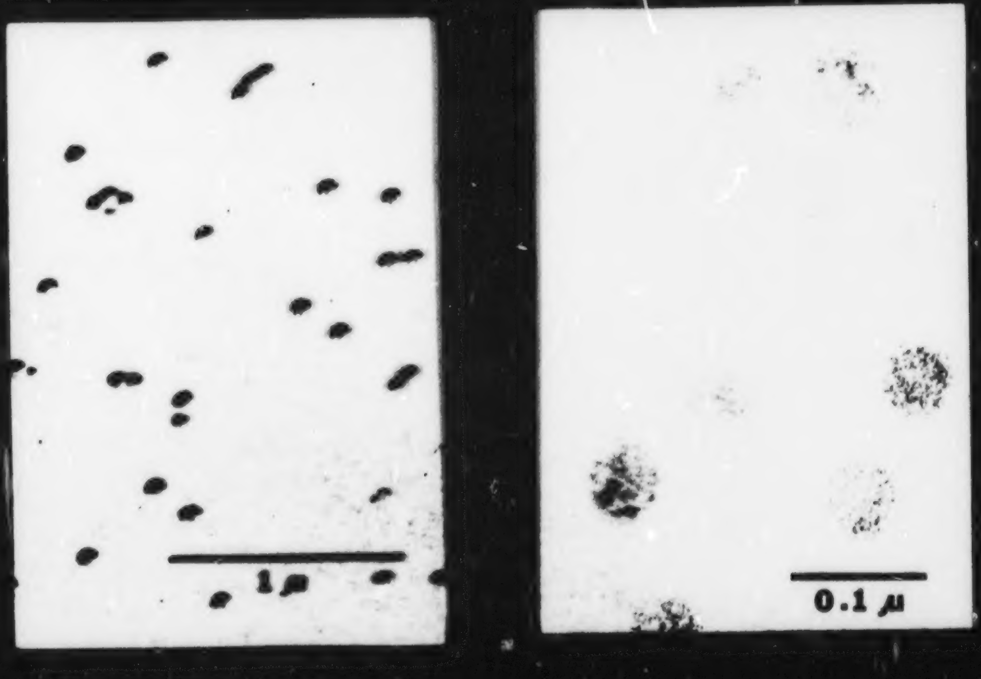

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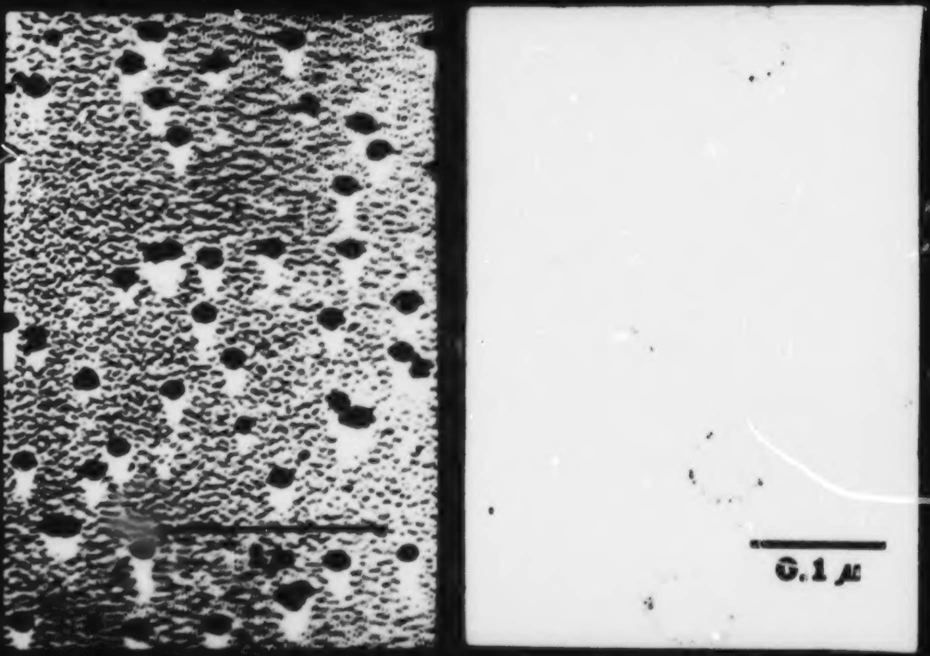

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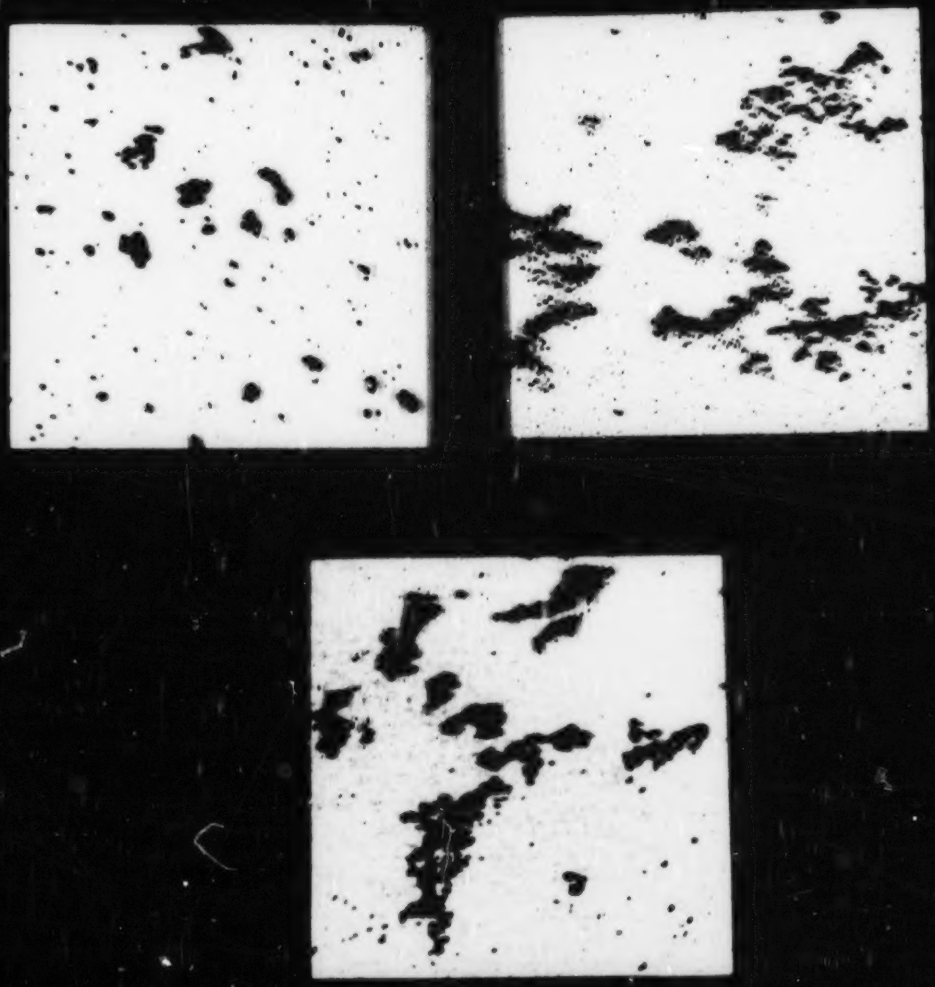

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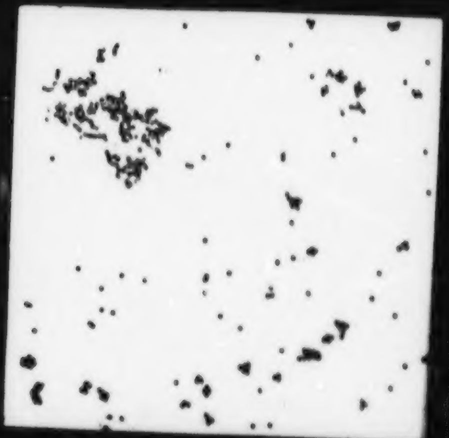

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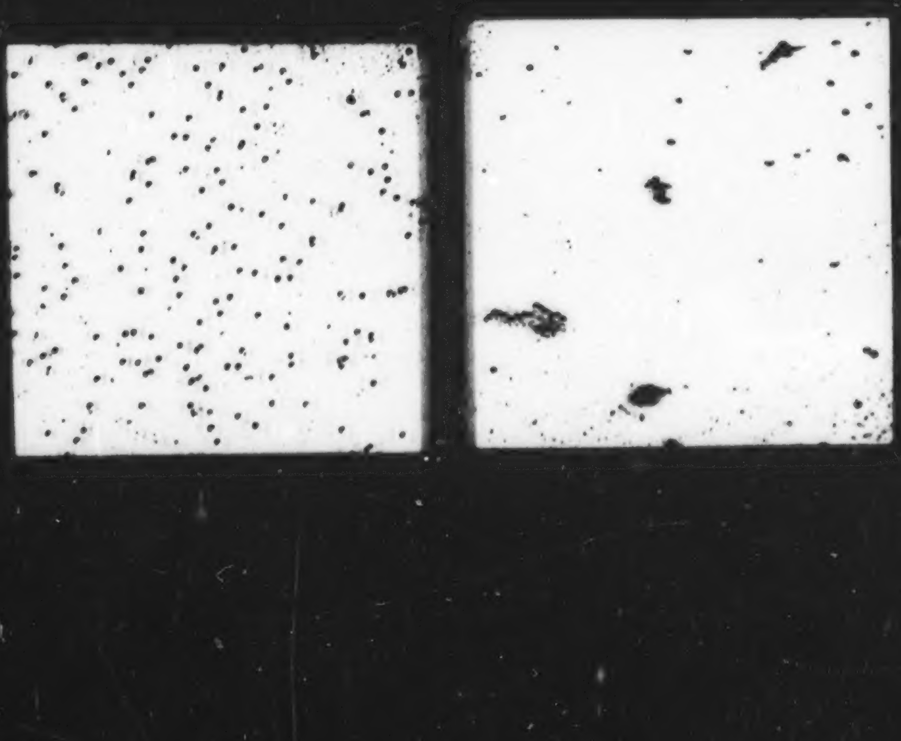

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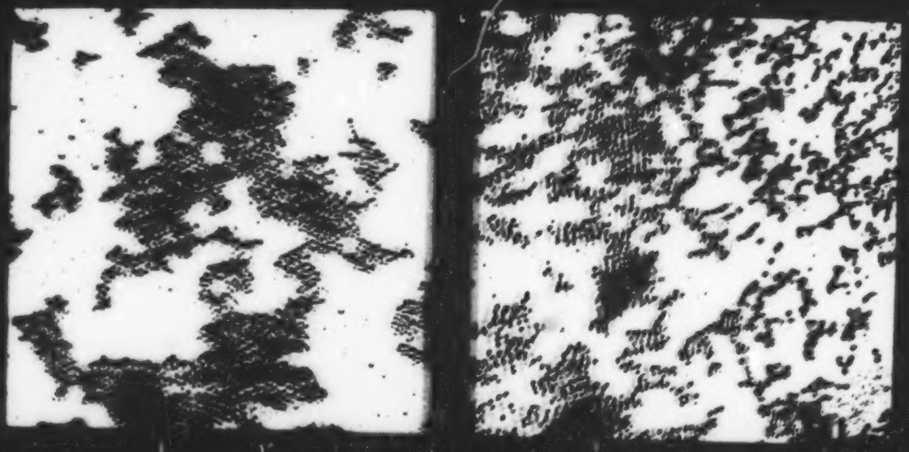

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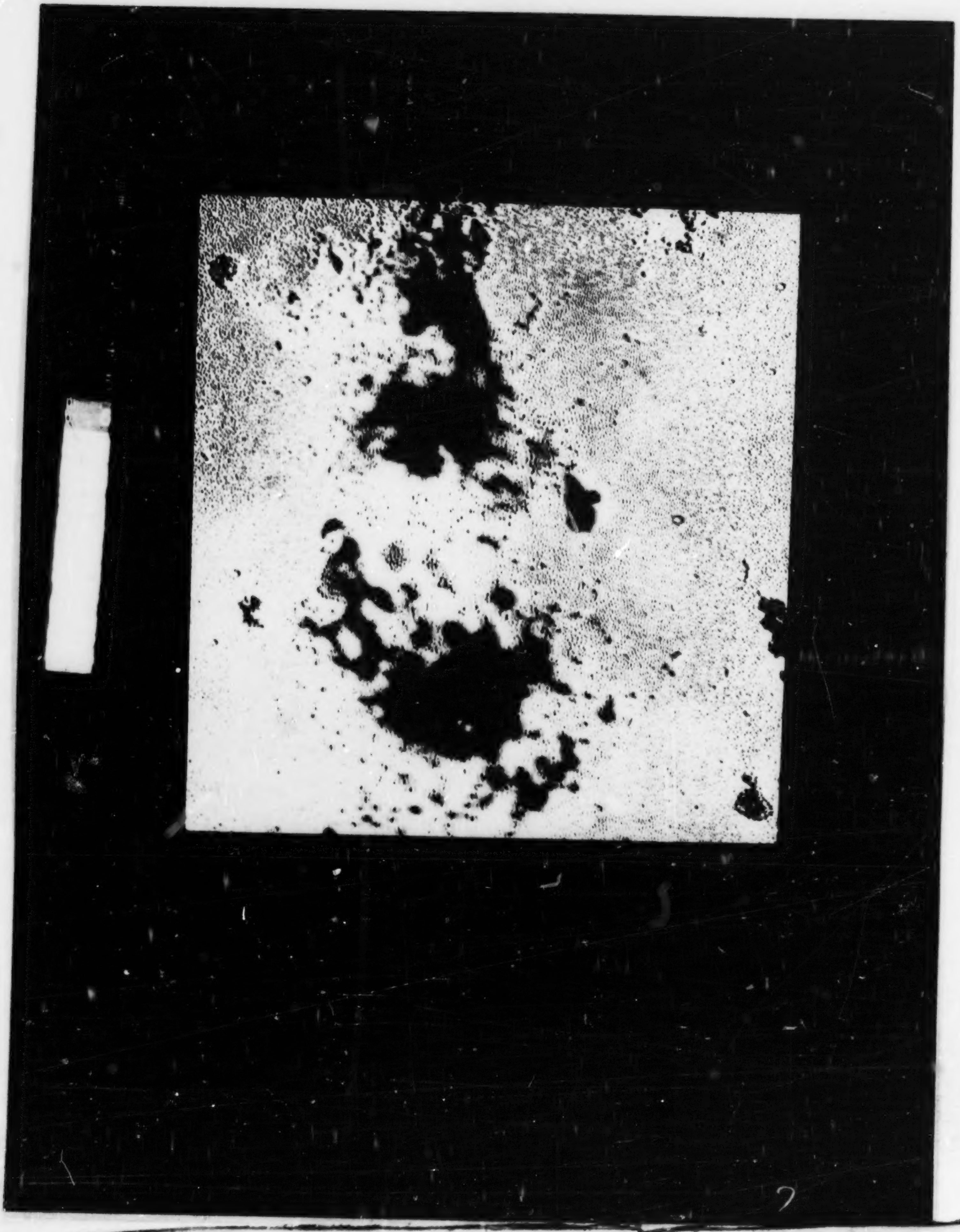

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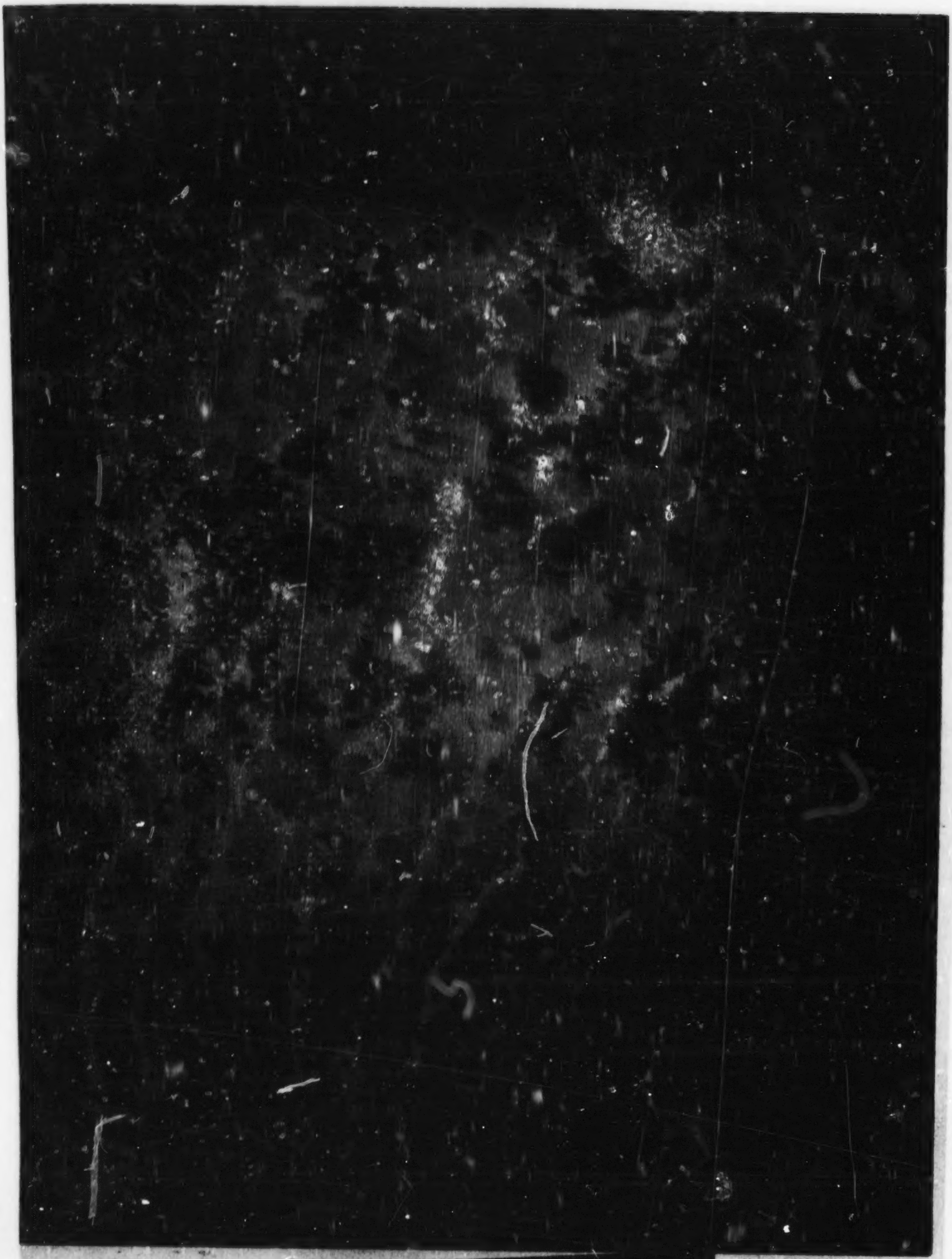

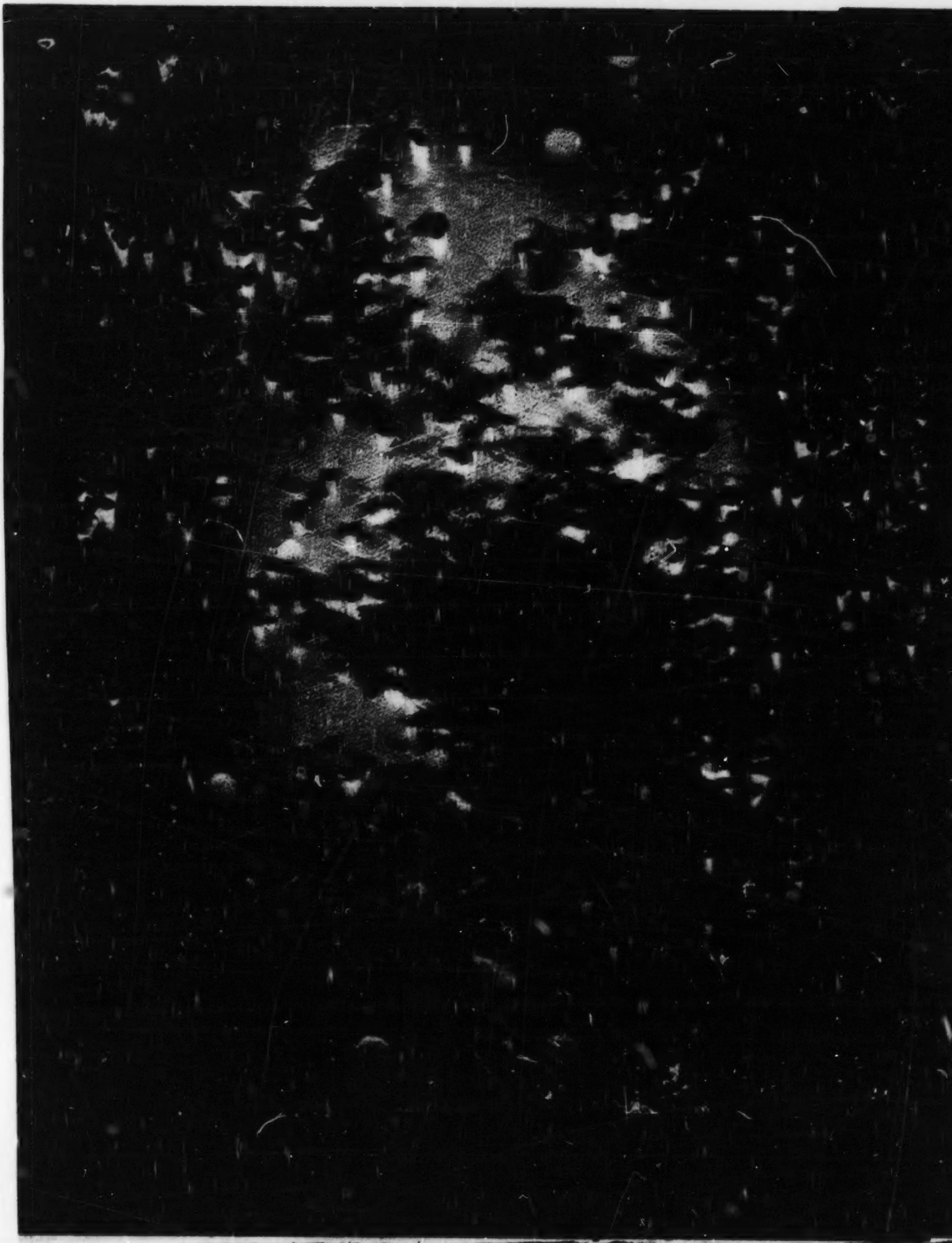

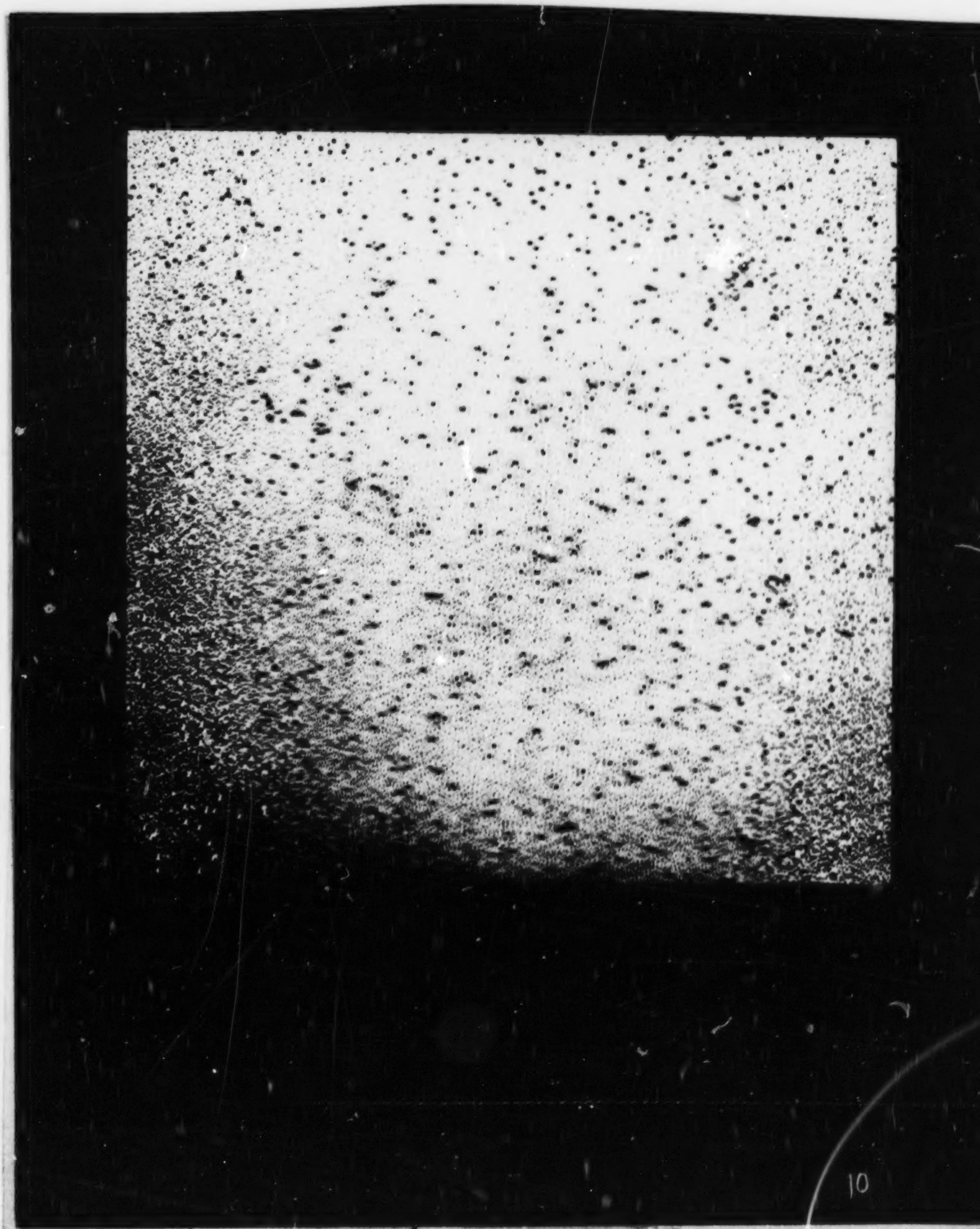

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